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Animal models of ovarian cancer

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Published: 07 October 2003

Reproductive Biology and Endocrinology 2003, **1**:67

Received: 28 June 2003
Accepted: 07 October 2003

This article is available from: http://www.RBEj.com/content/1/1/67

Abstract

Ovarian cancer is the most lethal of all of the gynecological cancers and can arise from any cell type of the ovary, including germ cells, granulosa or stromal cells. However, the majority of ovarian cancers arise from the surface epithelium, a single layer of cells that covers the surface of the ovary. The lack of a reliable and specific method for the early detection of epithelial ovarian cancer results in diagnosis occurring most commonly at late clinical stages, when treatment is less effective. In part, the deficiency in diagnostic tools is due to the lack of markers for the detection of preneoplastic or early neoplastic changes in the epithelial cells, which reflects our rather poor understanding of this process. Animal models which accurately represent the cellular and molecular changes associated with the initiation and progression of human ovarian cancer have significant potential to facilitate the development of better methods for the early detection and treatment of ovarian cancer. This review describes some of the experimental animal models of ovarian tumorigenesis that have been reported, including those involving specific reproductive factors and environmental toxins. Consideration has also been given to the recent progress in modeling ovarian cancer using genetically engineered mice.

Introduction

Despite improved knowledge of the etiology of ovarian cancer, aggressive cytoreductive surgery, and modern combination chemotherapy, there has been little change in the mortality statistics over the last 30 years, and approximately 60% of the women who develop ovarian cancer will die from their disease. Lack of an adequate screening test for early disease detection and the rapid progression to chemoresistance have prevented appreciable improvement in the five year survival rate of patients with ovarian cancer.

Experimental models for human diseases are of crucial importance not only to understand the biological and genetic factors that influence the phenotypic characteristics of the disease but to utilize as a basis for developing rational intervention strategies. Ovarian cancer cell lines derived from ascites or primary ovarian tumors have been used extensively and can be very effective for studying the processes controlling growth regulation and chemosensitivity. Our limited knowledge of the initiating events of ovarian cancer has restricted the development of models in which the early pathogenic events for ovarian cancer can be studied. However, there are a few animal models that develop ovarian tumors spontaneously, and others where the manipulation of various reproductive factors or exposure to environmental toxins have been shown to promote ovarian tumorigenesis. Finally, the recent

identification of promoters that can drive gene expression in the ovarian surface epithelium is providing new opportunities for the generation of genetically engineered mouse models of ovarian cancer. Here we describe some of the models that have been developed to investigate ovarian cell transformation.

Spontaneous and Non-epithelial Ovarian Tumorigenesis

There are few animal models that develop ovarian tumors spontaneously. Hens maintained under intensive egg-laying conditions develop ovarian adenocarcinomas; however such tumors are uncommon in hens less than 2 years of age [1]. Ovarian tumors will also arise spontaneously with age in some strains of mice [2], and in Wistar and Sprague-Dawley rats [3,4]. These tumors show a wide variety of histologic sub-types, including tubular adenoma, adenocarcinoma, papillary cystadenoma, mesothelioma, granulosa cell tumor, and polycystic sex cord/stromal tumor. However, the low incidence and/or the length of time required for the appearance of tumors in all of these models render them poorly feasible for experimental studies of ovarian carcinogenesis.

Some strains of mice, including C3HeB/Fe and C3HeB/De, show a high incidence of spontaneously occurring granulosa cell tumors and tubular adenomas [5]. Strain HAN:NMRI develop spontaneous Sertoli cell-like tumors and (DBA × Ce)F1 hybrids have a high incidence granulosa cell tumors [5]. Granulosa cell tumors also appear spontaneously at 4–6 weeks of age in SWR/J and in SWR/Bm inbred strain mice, with a maximum incidence reached by 10 weeks [6]. In some SWXJ strains, granulosa cell tumors occur spontaneously, and in others granulosa tumors can only be induced by treatment with dehydroepiandrosterone [7].

Spontaneous germ cell tumors are less common, but have been reported in LT/Sv and related strains of mice. These mice have a high frequency of spontaneous ovarian teratomas arising from follicular oocytes that undergo parthenogenetic activation. In some strains, this defect appears to be associated with an arrest of the oocytes at metaphase of meiosis I [8]. Teratomas arising from parthenogenetic activation of oocytes also occur in c-*mos*-deficient oocytes, which fail to maintain meiotic arrest after oocyte maturation [9,10].

Mice generated to be deficient in the tumor suppressor gene Lats1 exhibit a lack of mammary gland development, infertility and growth retardation. Accompanying these defects are hyperplastic changes in the pituitary and decreased serum hormone levels. The reproductive hormone defects of Lats1-/- mice are reminiscent of isolated LH-hypogonadotropic hypogonadism and corpus luteum insufficiency in humans. Lats1-/- mice develop soft-tissue sarcomas and ovarian stromal cell tumors [11].

The Ovarian Surface Epithelium

Although ovarian cancer in humans can arise from any of the cell types found in the ovary, almost 90% are derived from the ovarian surface epithelium (OSE) [12]. The OSE covers the entire ovarian surface, and varies morphologically from simple squamous to cuboidal to low pseudostratified columnar [13,14]. Embryologically derived from the mesodermal epithelium of the gonadal ridges, OSE cells are continuous with the flattened mesothelium of the peritoneum [15] and are separated from the underlying stromal compartment of the ovary by a basement membrane. Immunohistochemical staining has shown that OSE cells express cytokeratin, desmoplakin, transforming growth factor-α (TGF-α) and receptors for estrogen, progesterone and epidermal growth factor (EGF) [16–20]. Despite their rather unremarkable appearance *in vivo*, it is believed that OSE cells actively participate in the ovulatory process. Studies in rabbits and sheep have shown that OSE release proteolytic enzymes that degrade the basement membrane and the underlying apical follicular wall, weakening the ovarian surface to the point of rupture [21]. The OSE cells directly over the point of rupture undergo apoptotic cell death before ovulation [22] and the wound created at the ovulatory site surface is repaired by rapid proliferation of OSE cells from the perimeter of the ruptured follicle [23]. The biology, endocrinology and pathology of the ovarian surface epithelium have recently been reviewed in detail [24].

Although the ovarian surface is generally smooth in early reproductive life, with aging the ovary becomes more convoluted. Invaginations of the epithelium result in crypts or gland-like structures that can become pinched off to form epithelial inclusion cysts within the underlying stromal compartment [25]. This may occur following the postovulatory proliferation of OSE, follicular attrition, and/or from inflammation caused by carcinogens or chemical irritants like talcum powder [26]. The incidence of inclusion cysts increases with advancing age and are common in postmenopausal women. Although generally benign in nature, these epithelial rearrangements are widely thought to be the potential origin of many epithelial cancers. The more frequent appearance of epithelial invaginations and inclusion cysts in women with hereditary risk of ovarian cancer has strengthened this hypothesis [27]. In addition, some microscopic borderline and malignant tumors have been observed to arise directly within these sites, and they are often associated with dysplasia in similar sites elsewhere in the same or contralateral ovary [28,29].

Xenografts of OSE Cells Transformed *in vitro*

OSE cells have been implicated as the cell of origin for the majority of ovarian cancers based primarily on histological and immunohistochemical analyses of patient samples, but several recent experimental models manipulating these cells *in vitro* have provided additional support for this concept. Primary culture of human OSE was first reported by Auersperg et al. in 1984 [30], and her group has since developed several *in vitro* models of ovarian epithelial carcinogenesis. Introduction of Kirsten murine sarcoma virus into rat OSE cells results in endometrioid tumors following subcutaneous or intraperitoneal injection into immunosuppressed rats [31]. Transfection of SV40 T antigen early genes induces immortalization of human OSE cells that delays, but does not prevent, the senescence that normally occurs after a few passages [32]. Introduction of E-cadherin into these T antigen-immortalized cells induces epithelial differentiation [33] and the cells formed transplantable, invasive adenocarcinomas when injected into SCID mice [34]. In contrast to T antigen-immortalized cells, introduction of the human papilloma virus E6 and E7 genes into human OSE cells results in the spontaneous progression from a benign to invasive phenotype [35].

Unlike human OSE, rat and mouse OSE do not senesce. Rat OSE cells that have spontaneously immortalized but are not tumorigenic (eg. ROSE 199 cells; [36]) have been used in a variety of experiments, including some to characterize the cellular features when SV40 T antigen or H-*ras* is introduced into immortalized cells and following the formation of tumors when these cells are xenografted into nude mice [37]. Repeated subculture of rat and mouse OSE cells to maintain continued proliferation results in spontaneous malignant transformation, as characterized by loss of contact inhibition, substrate-independent growth and the ability to form tumors in nude mice [38,39]. In a variation of the above *in vitro* transformation approaches, Orsulic and colleagues used the RCAS retroviral vector to introduce oncogenes into OSE cells from transgenic mice bearing the RCAS receptor TVA and the cells were evaluated for tumorigenicity by injection into immune-deficient or syngeneic animals [40]. The investigators found that p53 deficiency in combination with two oncogenes from among C-*MYC*, *K-RAS*, or *AKT* were required to achieve transformation.

While these models allow an evaluation of oncogenes whose activation may contribute to the development of epithelial ovarian cancer, this approach does not allow the investigation of the early events in ovarian tumorigenesis inherent in mice when the tumors arise *in situ*. However, the establishment of *in vitro* models of normal and transformed OSE cells has provided the opportunity to use molecular approaches such as microarray or suppression subtractive hybridization to identify differential gene expression patterns that can distinguish normal OSE and ovarian cancer cells [41,42]. These data will be useful for the elucidation of molecular events associated with OSE cell transformation.

Xenografts of Cancer Cells

Xenograft models, where ovarian cancer cells have been injected either subcutaneously or into the peritoneal cavity have been used extensively for the testing of novel therapeutics or modified regimens for administration of standard chemotherapeutic drugs [43–45]. Some mouse models take advantage of the presence of a bursa, a sac-like structure that envelops rodent ovaries. For decades, researchers have used the intra-bursal space for transplants of xenografted ovaries, or to facilitate direct exposure of the ovary to various factors. For the generation of mouse models of ovarian cancer, the injection of ovarian cancer cells into the intra-bursal space results in tumor formation that can perhaps be viewed as more physiological (Figure 1), as the cancer cells are placed directly in the environment where ovarian tumors normally arise [46].

Reproductive Factors and Ovarian Tumorigenesis

Unlike most other cancers, the series of events involved in the initiation, progression and metastasis of ovarian cancer is not yet established. It is not clear if malignancies arise from benign or borderline tumors or if they develop *de novo* from the surface epithelium or inclusion cysts, as there is evidence for both [47]. The incidence of ovarian cancer climbs dramatically in women around the age at which they reach menopause. The reason for this is not clear, but two of the major changes associated with menopause form the foundation for hypotheses regarding the origin of ovarian tumors: 1) the depletion of oocytes or germ cells, which is the underlying cause of menopause, and 2) a significant increase in the pituitary's production of the gonadotropic hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), that arises as a consequence of the reduced follicular estrogen levels. In addition to the loss of germ cells and the associated alterations in hormone levels which normally occur at menopause, there are a number of non-menopausal factors that have been shown to have physiological relevance in epithelial ovarian tumorigenesis, including ovulation. Each of these will be discussed in the context of the animal models that have resulted from the experimental manipulations of these factors.

Ovulation

The "incessant ovulation hypothesis" proposes that continuous ovulation, with its successive rounds of surface rupture and OSE cell mitosis to repair the wound, renders the cells susceptible to malignant transformation [48].

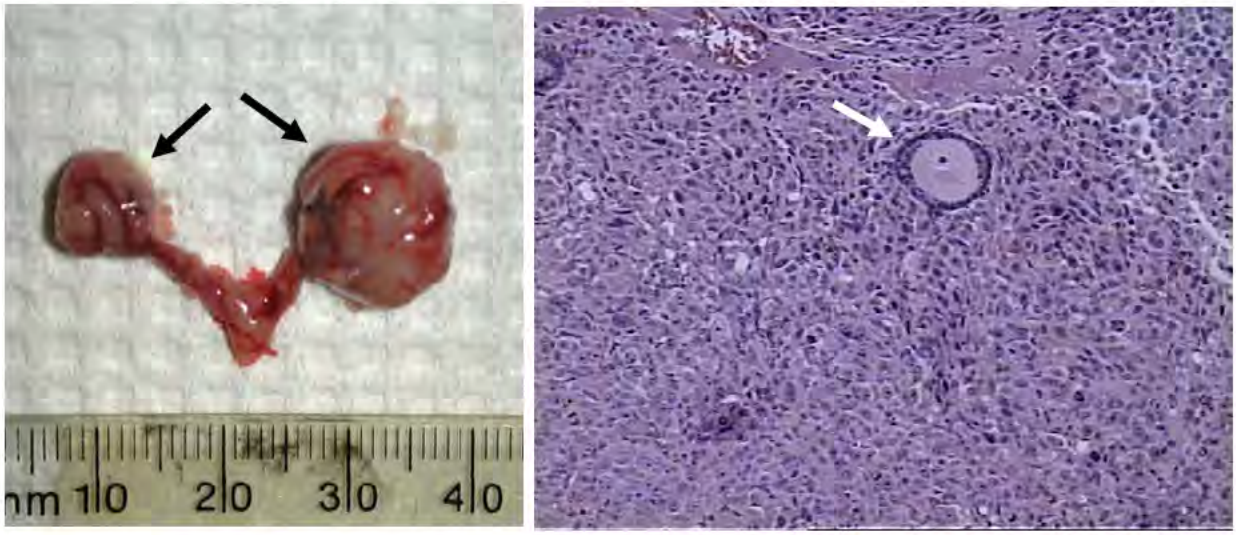

Figure 1
Development of ovarian tumors following injection of ES-2 ovarian cancer cells under the bursal membrane of nude mouse ovaries. Left figure- Proliferating cancer cells invade the normal tissue and increase the ovarian mass to diameters > 10-fold in size (indicated by arrows). Right figure- A single follicle containing a growing oocyte, indicated by an arrow, is clearly visible in the mass of tumor tissue.

Anecdotal support for this hypothesis comes from the observation that intensive egg-laying domestic hens frequently develop peritoneal carcinomata that is presumably of ovarian origin [1]. Epidemiological studies indicate that circumstances that decrease the number of ovulations, i.e., pregnancy, oral contraceptive usage, duration of lactation and early menopause, all substantially reduce the risk of ovarian cancer [49,50].

Inherent in the incessant ovulation hypothesis for ovarian cancer risk is the premise that repetitive damage of the OSE at ovulation and/or the subsequent mitotic repair following ovulation increases the risk of developing ovarian cancer. Experimental evidence to support the susceptibility of OSE cells to mutagenic events during mitosis is provided by studies showing that primary cultures of normal rat and mouse OSE cells which have been repeatedly subcultured to maintain continued proliferation acquire features associated with malignant transformation, including loss of contact inhibition, substrate-independent growth and the ability to form tumors in nude mice [38,39].

The risk generated by incessant ovulation may also be associated with the formation of epithelial cell-lined inclusion cysts that are frequently found in the ovarian stroma of perimenopausal women. As noted above, these inclusion cysts may form as a result of the process of ovulation and the pinching off of deep clefts [47]. In mice, the lifetime total number of ovulations is associated with a marked increase in OSE invagination and stratification [51], although the incidence of inclusion cysts was more related to age than to number of ovulations. Therefore, unlike in humans, an association between number of ovulations and ovarian cancer risk has not been demonstrated in rodents.

Gonadotropins

An alternative, but not mutually exclusive, hypothesis for the mechanism of ovarian carcinogenesis proposes that the development of ovarian tumors is related to excessive gonadotropin production associated with the onset of menopause or premature ovarian failure [52]. The median age for epithelial ovarian cancer is 60–65 years, with only 10–15% of the tumors appearing in premenopausal women [53]. Serum FSH and LH levels reach their peak during perimenopausal and postmenopausal years and remain elevated thereafter [54]. High circulating levels of pituitary gonadotropins may increase the risk of ovarian cancer by stimulating the growth of ovarian epithelial

cells, since normal human OSE cells and epithelial inclusions have been found to express receptors for FSH [55] and LH/hCG [56]. Enhanced cell proliferation in response to FSH and/or LH/hCG has been reported for primary cultures of rabbit [57], mouse [58] and human [56] OSE cells. Schiffenbauer and colleagues [59] found that human epithelial ovarian cancers progressed faster in ovariectomized mice due to elevated FSH and LH levels, which promoted increased vascular endothelial growth factor expression and tumor neovascularization.

The gonadotropin theory of ovarian tumorigenesis suggests that elevated gonadotropin concentrations contribute to the development of ovarian tumors. This theory is based on the initial observation of Biskind and Biskind in 1944 [60] who reported that transplantation of ovaries into the splenic pulp of adult rats led to the development of ovarian tumors. The tumorigenesis was attributed to inactivation of estrogen in the liver, and the consequent elevation of gonadotropin levels due to the lack of steroid feedback on the pituitary. Several transgenic or knockout animal models in which gonadotropin levels are elevated also result in ovarian tumorigenesis. For example, when inhibin, the ovarian protein that inhibits the production of FSH, is made deficient in mice, gonadal stromal tumors arise [61]. Transgenic mice generated to have chronic LH hypersecretion develop granulosa cell tumors or luteomas, depending on the background strain [62,63]. Mice with disruption of the FSH receptor are acyclic and sterile, with very small, underdeveloped ovaries; they exhibit hypergonadotropic-hypogonadism with high levels of circulating FSH and LH similar to the postmenopausal state in women. By 12 months, more than 92% of these animals developed various kinds of ovarian pathology, including neoplasms of sex cord-stromal type as well as cysts, suggesting that FSH receptor insensitivity in the face of prolonged elevated levels of gonadotropins may be contributing to the development of ovarian granulosa or stromal tumors [64]. None of the animal models with targeted manipulation of gonadotropin secretion or action appear to promote ovarian epithelial tumorigenesis.

Steroid hormones

In the developing fetal ovary, marked OSE cell proliferation occurs at 16 to 20 weeks of gestation, coincident with the appearance of steroid-producing cells in the ovarian cortex [65]. Adult human OSE cells express receptors for estrogen, progesterone and androgens [66,67], and human OSE cell proliferation can be stimulated by androgens [68]. In contrast, human OSE cells in culture are reportedly unaffected by estradiol or progesterone [66], which would suggest that these steroid hormones do not have a significant role in ovarian tumorigenesis. However, a recent study has found that menopausal women who have taken hormone replacement therapy using estrogen only are at an increased risk of ovarian cancer [69]. In animals, continuous exposure to estradiol stimulates sheep OSE cell proliferation [70], while in guinea pigs and rabbits, it results in the formation of a papillary ovarian surface resembling human serous neoplasms of low malignant potential [71,72]. The mechanisms by which estrogen may contribute to ovarian cancer risk is unknown, but could be direct action on the OSE cells, or may be indirect, as estrogen reduces GnRH receptor expression in both OSE and ovarian cancer cells, thereby suppressing the growth inhibitory effects of GnRH [73]. Estrogen also modulates levels of hepatocyte growth factor which stimulates OSE cell growth [74].

A number of studies, largely epidemiological, provide support for the hypothesis that androgens are involved in ovarian carcinogenesis. Over 80% of tumors express AR [75] and an increased risk of ovarian cancer was found in women with elevated circulating levels of androgens [76]. Testosterone-stimulated growth of OSE cells in guinea pigs caused the formation of benign cysts, small adenomas in the ovarian parenchyma, and papillomas on the ovarian surface [77]. Androgens may promote ovarian tumorigenesis in part by decreasing TGF-β receptor levels, thereby allowing ovarian cancer cells to escape TGF-β growth inhibition [78].

Germ cell deficiency/depletion

Aging and hereditary risk are associated with a more frequent incidence of epithelial invaginations and inclusion cysts, putative preneoplastic precursor lesions, but the underlying mechanisms for these epithelial-stromal rearrangements are unknown. OSE cell hyperplasia with stromal invasion has been reported in a diverse array of experimental situations, all of them involving loss of germ cells and consequent failure of follicle development. For example, mutations at the *W* (*Kit*) or *Sl* (*Kitl*) loci result in sterility by preventing the normal proliferation and migration of germ cells during fetal development [79]. Germ cell deficiency *in vivo*, as is found in W^x/W^v mice, results in bilateral ovarian tubular adenomas in more than 95% of the animals by 5 months of age [80,81]. The tumors arise from interstitial cell hyperplasia, with proliferation and invasion of the ovarian surface epithelium into the stromal compartment of the ovary. Invasive epithelial tubules are also found in Sl/Sl^t germ cell deficient mice by 7 months of age [82], and mice heterozygous for the Sl^d mutation, which carries a splicing defect, develop papillary structures and epithelial invaginations (Figure 2), similar to that seen in women [26]. Likewise, female mice homozygous for the germ cell deficient (*gcd*) mutation enter reproductive senescence prematurely due to a dearth of germ cells. By one year of age, 56% of homozygotes have developed ovarian tubulostromal adenomas while wild-type littermates are phenotypically normal [83].

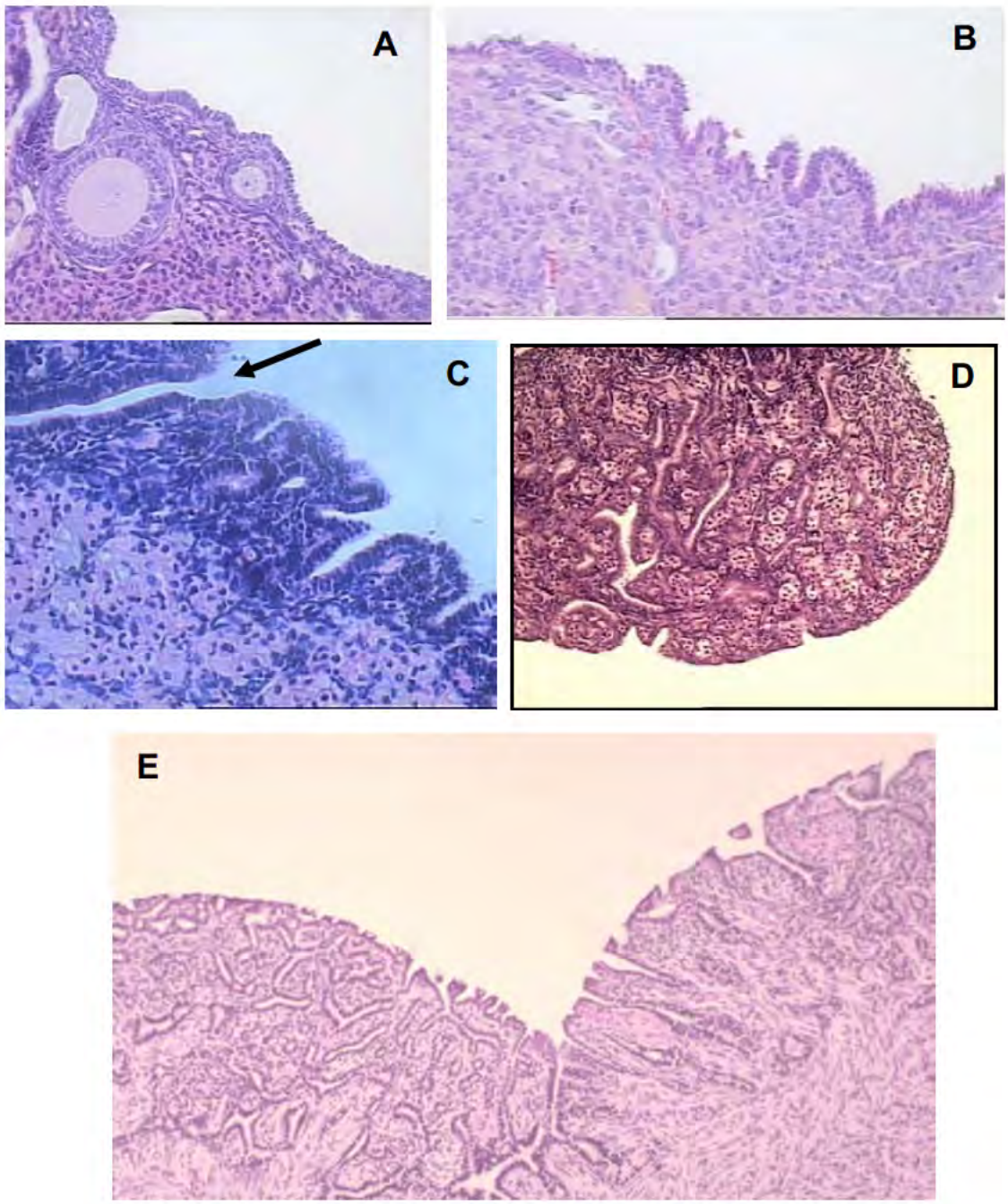

Figure 2
Morphology of the ovarian surface epithelium in wild-type (A; 12 months), *Sl*^{d} heterozygous (B, C; 12 months) and homozygous (D; 6 months) mice. Ovaries from wild-type mice contain developing follicles and a covering layer of columnar OSE. In 12-month-old *Sl*^{d} heterozygous mice, there is a depletion of follicles, and the ovarian surface has become very convoluted (B), with this papillary surface sometimes leading to deep invaginations, as indicated by the arrow (C). By 6 months of age, the ovaries of homozygous *Sl*^{d} mice are completely abnormal, with no recognizable ovarian structures, and are composed primarily of invasive epithelial tubules. (E) Human ovarian papillomatosis, for comparison.

Therefore, it appears that oocyte depletion is associated with formation of epithelial structures that resemble the preneoplastic lesions in human ovaries.

Experimental ovarian tumorigenesis has been investigated in inbred and hybrid strains of mice and induced by a diversity of mechanisms including X-irradiation, oocytotoxic xenobiotic chemicals, ovarian grafting to ectopic or orthotopic sites, neonatal thymectomy, genetic defects reducing germ cell populations, and aging [reviewed in [84]]. While germ cell deficiency seems to be a required element for the development of epithelium-derived adenomas, the mechanisms by which germ cell loss contributes to tumorigenesis in these models remain unclear. Ovarian follicles do not develop in the absence of oocytes, indicating that the oocyte directs the development of follicles. Pathogenetic factors that prematurely destroy or diminish the numbers of germ cells lead to failure in follicle development and a resulting decrease in sex steroid hormone secretion (notably estradiol) leading to a compensatory over-production of pituitary gonadotropins, which places the ovary at an increased risk to develop tumors. Therefore oocyte depletion, similar to that which occurs naturally by the time of menopause, may be a contributing factor to the oncogenic behavior of the surface epithelial cells.

The intense proliferation of OSE and stromal (interstitial) cells with the development of unique tubular adenomas in response to sterility seems to require both the lack of germ cells/follicles and the increased production of gonadotropins. Elevated gonadotropins alone resulted in granulosa cell tumors or luteomas [62,63]. Oocyte destruction by gamma irradiation in hypogonadal mice deficient in gonadotropins did not result in the development of tubular adenomas [85]. Similarly, the experimental suppression of gonadotropin levels in W^x/W^v mice was sufficient to prevent the development of ovarian tubular adenomas from the surface epithelium [86], suggesting that both oocyte loss/destruction and elevated gonadotropins are necessary for epithelial tumorigenesis.

Environmental Carcinogens

Although the more established hypotheses that have been proposed to explain increased risk of developing ovarian cancer are related to the number of ovulations or to increased hormone levels, there are additional risk factors that have been identified, including a number of environmental carcinogens. While these factors have been reported to have effects on the ovarian surface epithelium, they are usually also associated with follicular destruction and/or ovotoxicity, so indirect actions due to altered gonadotropin levels cannot be eliminated. Use of perineal talc has been identified as a risk factor, possibly due to its ability to ascend the genital tract and affect the ovarian surface [87]. Indeed, direct exposure of rat ovaries to talc results in focal areas of papillary change in the ovarian surface epithelium, as well as ovarian cysts [88]. Exposure of rhesus and cynomolgus monkeys to the environmental pollutant, hexachlorobenzene results in both reproductive failure and notable alterations in the size, shape and degree of stratification of the OSE cell layer [89]. More recent studies have shown that the insecticide methoxychlor increases both the height of the OSE cell layer and the percentage of atretic follicles in exposed mice [90]. In rodent studies, ovarian toxicity and/or carcinogenicity has been documented for at least eight chemicals that result in follicular necrosis, tubular hyperplasia, granulosa cell tumors and benign mixed tumors [91,92]. N-ethyl-N-nitrosourea administered to rats intraperitoneally or transplacentally increases the incidence of ovarian tubular adenomas [93]. The mechanisms by which these environmental carcinogens enhance the risk of ovarian tumors remain unexplored.

Transgenics and Targeted Approaches to Transform the Ovarian Epithelium

The ideal model to investigate the pathogenic events associated with early ovarian tumorigenesis would be a mouse model in which the tumor arises directly from the OSE cells. This model would differ from current xenograft models in that transgenic mice with defined genetic lesions could be studied at various stages as they inevitably develop ovarian cancer *in situ*. In addition, the development of a genetic model would permit the direct testing of oncogenes and tumor suppressors for their contribution to the initiation and progression of overt malignancies in the mouse ovary. Finally, a number of different factors could be altered such as the genetic background of the mouse strain, the frequency of ovulation and the levels of various hormones to determine their impact on the development of tumors in the susceptible transgenic mouse line.

One approach to alter gene expression directly in the OSE cells would be to take advantage of the fact that these cells readily take up and express genes delivered by intra-bursal injection of adenoviruses [94,95]. This method has the potential advantage of mimicking somatic mutations that contribute to early ovarian tumorigenesis. One recent report used intra-bursal adenovirus delivery and Cre-loxP mediated gene inactivation to render OSE cells deficient in two key tumor suppressor genes: p53 and Rb [95]. The p53 tumor suppressor gene is the most frequently mutated gene in human neoplasms. Mutations and/or over-expression of p53 have been described in 26–62% of ovarian cancers, particularly serous ovarian carcinomas [reviewed in [96]]. Aberrations in the Rb pathway have been reported [97]; however, direct evidence for their contribution to ovarian epithelial tumorigenesis is lacking. In

this model, recombinant adenovirus expressing Cre was injected under the ovarian bursal membrane of double transgenic mice bearing floxed copies of *p53* and *Rb*. Concurrent inactivation of p53 and Rb was sufficient for reproducible induction of ovarian epithelial carcinogenesis in mice homozygous for the conditional alleles. While less than 15% of mice with inactivation of either *Rb* or *p53* developed tumors, 33 of 34 mice with deficiencies in both genes succumbed to their ovarian cancers at a median of 227 days, with 24% having abdominal ascites.

The major impediment to the development of transgenic models of ovarian cancer is the lack of specific promoters able to direct gene expression to OSE cells. Previous models of ovarian cancer have resulted in granulosa cell tumors using promoters, such as inhibin-alpha subunit promoter, that are active in this cell type to drive the expression of the large T antigen of SV40 [98,99]. Recent studies have identified two other promoters that may prove to be useful for the generation of transgenic models of ovarian cancer. The Ovarian Specific Promoter (OSP-1) was developed from a retrovirus-like element specifically expressed in the rat ovary. The promoter drives gene expression specifically in normal and neoplastic ovarian epithelial cells [100] and expression of *lacZ* driven by OSP-1 in transgenic mice was restricted to the ovary as determined by X-gal staining of multiple organs [101]. Immunohistochemical detection of β-galactosidase showed *lacZ* expression mainly in the granulosa cells and ovarian surface epithelial cells. However, transgenic mice in which OSP-1 drives the expression of the early region of SV40 virus developed tumors in a variety of tissues, including unilateral granulosa cell tumors in two of three female founder mice. Thus, although transcription from the OSP-1 promoter occurs predominantly in the ovary, this promoter is sufficiently "leaky" in cells in other tissues to permit their tumorigenic conversion by SV40 TAg.

The first transgenic model of epithelial ovarian cancer was recently reported and used the upstream region of the Mullerian inhibitory substance type II receptor (*MISIIR*) gene to drive tissue-specific expression [102]. MISIIR is a single transmembrane serine/threonine kinase that shares homology with the TGFβ-receptor [103,104]. Expression of *MISIIR* has been reported to be restricted to mesenchymal cells surrounding the Mullerian duct during embryogenesis, tubular and follicular structures of fetal gonads, Sertoli and Leydig cells of adult testis, and granulosa cells of adult ovary [103,105,106]. More recently, expression of *MISIIR* in established human ovarian cancer cell lines as well as cell lines derived from the ascites of patients with ovarian carcinomas has been demonstrated [107]. Transgenic mice in which the 5' upstream regulatory sequences of the mouse *MISIIR* gene were used to target expression of the SV40 *TAg* specifically to the epithelium of the female mouse reproductive tract, including the OSE, developed ovarian carcinomas with metastatic spread to peritoneal organs by 3 months of age. Female transgenic mice developed bilateral ovarian tumors in ~50% percent of cases. Histologically, these tumors were poorly differentiated carcinomas with occasional cysts and papillary structures present at the surface of the ovary. These tumors disseminated intraperitoneally, invaded the omentum and formed ascites in a manner that resembles human ovarian carcinomas. The demonstration that the *MISIIR* promoter can be used successfully to drive gynecological tissue-specific transgene expression in mice and that this often results in the formation of ovarian carcinoma offers very promising opportunities for testing the efficacy of chemotherapeutic and chemopreventive agents in a heritable model of epithelial ovarian cancer.

Conclusions

The two most pressing problems in the management of ovarian cancer are the lack of adequate diagnostic or screening strategies, and the recurrence of disease that is often chemoresistant. In part, the deficiency in diagnostic tools is due to the lack of markers for the detection of preneoplastic or early neoplastic changes in the OSE cells. The generation of animal models in which OSE cells undergo neoplastic transformation *in vivo* will provide much-needed opportunities to investigate the cellular and molecular changes associated with the initiation of OSE cell transformation, as well as to provide models in which prevention, diagnostic, screening and therapeutic strategies can be developed.

Acknowledgements

The authors wish to thank Dr. Ken Garson for critical review of this manuscript and Dr. Thomas Hamilton, Fox Chase Cancer Center, for providing the photograph showing human papillomatosis. Research was supported by grants from the National Cancer Institute of Canada (BCV) and the National Institutes of Health (BCV; Dr. Thomas Hamilton, Principal Investigator), a scholarship from the Canadian Institutes of Health Research (TJS) and a fellowship from a partnership of the Mitchell Family Fund, the National Ovarian Cancer Association and Cancer Care Ontario (JFE).

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Exhibit 105

UNITED STATES DISTRICT COURT
DISTRICT OF NEW JERSEY

IN RE JOHNSON & JOHNSON TALCUM POWDER PRODUCTS MARKETING, SALES PRACTICES, AND PRODUCTS LIABILITY LITIGATION ***THIS DOCUMENT RELATES TO ALL CASES***	**MDL NO. 16-2738 (FLW) (LHG)**

RULE 26 EXPERT REPORT OF DR. GHASSAN M. SAED

Date: November 16, 2018

Dr. Ghassan M. Saed

Molecular basis for the association of talcum powder use with increased risk of ovarian cancer.

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Qualifications

In this report, I describe the role of oxidative stress in the pathogenesis and behavior of ovarian cancer, as well as describe the biological effects of talcum powder on normal ovarian and fallopian tube cells, macrophages, and ovarian cancer cells.

I am an Associate Professor with tenure at Wayne State University in Detroit, Michigan, where I am Director of Ovarian Cancer Research. I am a faculty member in the Departments of Obstetrics & Gynecology, Cell Biology, and Anatomy & Physiology at Wayne State School of Medicine. I am also a Member of the Karmanos Cancer Institute, Molecular Biology and Genetics Program.

I received a Ph.D. in Molecular Biology at the University of Essex, Colchester, England in 1986. My postgraduate training included a Fellowship in Immunopathology at the University of Michigan, Ann Arbor from 1992-1993 and a Fellowship in Molecular Biology at the Henry Ford Hospital in Detroit, Michigan from 1988-1990. I joined the faculty at Wayne State School of Medicine in 1998.

My laboratory investigates the role of oxidative stress in the pathogenesis of ovarian cancer. This concentration arose from my original research that focused on the molecular mechanisms involved in the pathogenesis of tissue fibrosis and the need to compare the effects of oxidative stress on a malignant overgrowth versus a benign overgrowth, specifically postoperative adhesions.

My research in ovarian cancer has resulted in the identification of biomarkers for assessing the progression and metastasis of ovarian cancer. The major outcome of my work with fibrosis and postoperative adhesions, in addition to the development of the ex-vivo model for adgestiosn, was the development and characterization of the adhesion phenotype in cell culture. Additionally,

the cell culture system was used to test the hypothesis that hypoxia is the trigger for the development of postoperative adhesions.

I have taught numerous undergraduate, graduate, medical students, residents, and fellows. Many of these have received research awards, published important papers, and accepted prestigious academic faculty positions. I have been the recipient of national and international grants and contracts from organizations including the American Association for Cancer Research (AACR), NIH/NICHD, U.S. Department of Defense, the Ovarian Cancer Research Fund Alliance, the Michigan Ovarian Cancer Alliance, and other ovarian cancer foundations. I have been a prolific publisher and presenter at scientific meetings. I have been an author on 136 original studies published in peer-reviewed journals with additional review articles, and book chapters. Recently, I published a review article in the journal, Gynecologic Oncology titled, "Updates of the role of oxidative stress in the pathogenesis of ovarian cancer" and a textbook chapter titled "New insights into the Pathogenesis of Ovarian Cancer: Oxidative Stress" summarizing my research in this area. My CV is attached as Exhibit A. In addition to the references included in this report, the materials I reviewed are attached as Exhibit B. My fees and prior testimony are attached as Exhibit C.

Ovarian cancer

Ovarian cancer is the most lethal gynecologic malignancy and ranks fifth in cancer deaths among women diagnosed with cancer[1]. Epithelial ovarian cancer (EOC) has long been considered a heterogeneous disease with respect to histopathology, molecular biology, and clinical outcome[1,2]. It comprises at least five distinct histological subtypes, the most common and well-studied being high-grade serous ovarian cancer. In the last decade, researchers have proposed the theory that many ovarian cancers arise from the distal fallopian tubes. For this reason, as well as the similarities in pathogenesis, presentation, treatment, and prognosis, fallopian tube, ovarian, and

peritoneal cancer are generally treated as a single entity. Although surgical techniques and treatments have advanced over the years, the prognosis of EOC remains poor, with a 5-year survival rate of 50% in advanced stage [2]. This is largely due to the lack of early warning symptoms and screening methods, and the development of chemoresistance [1,2]. Ovarian cancer is known to be associated with germline mutations in the BRCA1 or BRCA2 genes, but with a rate of only 20-40%, suggesting the presence of other unknown mutations in other predisposition genes [3]. Additional genetic variations including single nucleotide polymorphisms (SNPs) have been described to act as low to moderate penetrant alleles that contribute to ovarian cancer risk [3,4]. Non-synonymous SNPs substitute encoded amino acids in proteins and are more likely to alter the structure, function, and interaction of the protein [4]. The pathophysiology of EOC is not fully understood but has been strongly associated with inflammation and the resultant oxidative stress[5].

Oxidative stress

Homeostasis, the balance between the production and elimination of oxidants, is maintained by mechanisms involving oxidants and antioxidant enzymes and molecules. If this balance is altered, it leads to an enhanced state of oxidative stress that alters key biomolecules and cells of living organism [5]. Oxidant molecules are divided into two main groups; oxygen-derived or nitrogen-containing molecules. Oxygen-derived molecules, also known as reactive oxygen species (ROS), include free radicals such as hydroxyl ($HO^{\bullet}$), superoxide ($O_2^{\bullet -}$), peroxyl ($RO_2^{\bullet}$), and alkoxyl ($RO^{\bullet}$), as well as oxidizing agents such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3), and singlet oxygen ($^1O_2$) that can be converted to radicals [5,6]. Nitrogen containing oxidants, also known as reactive nitrogen species (RNS), are derived from nitric oxide (NO) that is produced in the mitochondria in response to hypoxia [5]. Exposure to inflammation, infection, carcinogens, and toxicants are major sources of ROS and RNS, *in vivo* [5-8]. Additionally,

RNS and ROS can be produced by various enzymes including cytochrome P450, lipoxygenase, cyclooxygenase, nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase complex, xanthine oxidase (XO), and peroxisomes (Figure 1) [5,7,9].

To maintain the redox balance, ROS and RNS are neutralized by various important enzyme systems including superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), glutathione (GSH), thioredoxin coupled with thioredoxin reductase, glutaredoxin, glutathione peroxidase (GPX), and glutathione reductase (GSR) [6]. Superoxide dismutase is known to convert $O_2^{\bullet -}$ to H_2O_2, which is then converted to water by CAT. Glutathione S-transferase is involved in detoxification of carcinogens and xenobiotics by catalyzing their conjugation to GSH that will aid in expulsion from the cell [6]. Indeed, the GSH-to-oxidized-GSH (GSH/GSSG) ratio is a good indicator of cellular redox buffering capacity [10,11]. Under enhanced oxidative stress, the GSH/GSSG complex is known to stimulate the activity of the GS-X-MRP1 efflux pump, which removes toxins from cells. This mechanism has been investigated in the development of resistance to chemotherapeutic drugs [10,11].

Ovarian Cancer Cells Manifest a Persistent Pro-Oxidant State

Recent evidence demonstrates that oxidative stress is a critical factor in the initiation and development of several cancers, including ovarian cancer [12,13]. Consistently, it has been reported that ovarian cancer patients manifest significantly decreased levels of antioxidants and higher levels of oxidants [12-17]. An enhanced redox state, resulting from increased expression of key pro-oxidant enzymes and decreased expression of antioxidant enzymes, has been extensively described in epithelial ovarian cancer (EOC) [16-18]. My laboratory has previously reported that MPO, a hemoprotein present solely in myeloid cells that acts as a powerful oxidant, and iNOS, a key pro-oxidant enzyme, are highly expressed and co-localized to the same cell in EOC cells [17]. These two

enzymes, MPO and iNOS, work together to inhibit apoptosis, a hallmark of ovarian cancer cells. Apoptosis, or programmed cell death, refers to the normal and controlled death of cells. Myeloperoxidase acts as powerful oxidant enzyme in EOC cells through the utilization of nitric oxide (NO) produced by iNOS as a one-electron substrate generating NO^+, a labile nitrosylating species[19-21]. My laboratory was the first to report that MPO was expressed by EOC cells and tissues[17]. Silencing MPO gene expression utilizing MPO specific siRNA induced apoptosis in EOC cells through a mechanism that involved the S-nitrosylation of caspase-3 by MPO[17]. Additionally, there is compelling evidence that MPO serves as a source of free iron under oxidative stress, where both NO^+ and superoxide are elevated[19]. Iron reacts with hydrogen peroxide (H_2O_2) and catalyzes the generation of highly reactive hydroxy radical ($HO^\bullet$), thereby increasing oxidative stress, which in turn increases free iron concentrations by the Fenton and Haber–Weiss reaction[19,21]. Additionally, my laboratory has highlighted the potential benefits of the combination of serum MPO and free iron as biomarkers for early detection and prognosis of ovarian cancer[14]. EOC cells are also characterized by enhanced expression of NAD(P)H oxidase, a potent oxidant enzyme that is known to be the major source of $O_2^{\bullet-}$ in the cell. Such high levels of $O_2^{\bullet-}$ combined with significantly high levels of NO generates peroxynitrite, another powerful nitrosylation and nitration agent, which modifies proteins and DNA structure and function in cells [22].

A reliable screening and detection method based on molecular profiles for ovarian cancer has not yet been developed because the disease exhibits a wide range of morphological, clinical and genetic variations during its progression. The search for non-invasive, cost-effective ovarian cancer biomarker tests has been ongoing for many years. Immunizations of mice with ovarian cancer cells has led to hybridoma validation by ELISA, while flow cytometry analysis permitted the discovery of cancer antigen (CA)-125 (the only marker currently used in clinical practice) and

mesothelin [23]. Furthermore, the screening of an array of 21,500 unknown ovarian cDNAs hybridized with labeled first-strand cDNA from ten ovarian tumors and six normal tissues led to the discovery of human epididymis protein 4 (HE4) [24]. Most interestingly, HE4 is overexpressed in 93% of serous and 100% of endometrioid EOCs, and in 50% of clear cell carcinomas, but not in mucinous ovarian carcinomas [25]. Thus, HE4 was identified as one of the most useful biomarkers for ovarian cancer, although it lacked tissue-specificity [24,26-28]. Secreted HE4 high levels were also detected in the serum of ovarian cancer patients [29]. Additionally, combining CA-125 and HE4 is a more accurate predictor of malignancy than either alone [30-32]. The discovery of MPO expression in ovarian EOC cells and tissues was surprising, as it is only expressed by cells of myeloid origin. Intriguingly, my laboratory has previously reported that the combination of serum MPO and free iron may serve as biomarkers for early detection of ovarian cancer [14].

Common Polymorphisms in Redox Enzymes are Associated with Ovarian Cancer.

A single nucleotide polymorphism (SNP) occurs as a result of gene point mutations with an estimated frequency of at least one in every 1000 base pairs that are selectively maintained and distributed in populations throughout the human genome [33]. An association between common SNPs in oxidative DNA repair genes and redox genes with human cancer susceptibility has been established [7]. Common SNPs in the redox enzymes are known to be strongly associated with an altered enzymatic activity in these enzymes, and helps explain the enhanced redox state that has been linked to several malignancies, including ovarian cancer [12,16]. Additionally, it helps explain the observation of significantly decreased apoptosis and increased survival of EOC cells [17]. It is therefore critical to determine the exact effect of common SNPs in various redox enzymes on all process involved in the development of the oncogenic phenotype [34-37]. Such studies can be linked to other studies focusing on determining the effects of genes involved in carcinogen metabolism

(detoxification and/or activation), redox enzymes, and DNA repair pathways[36]. Numerous SNPs associated with change of function have been identified in antioxidant enzymes including *CAT*, *GPX1*, *GSR*, and *SOD2* [35,37]. Additionally, the association between genetic polymorphisms in genes with anti-tumor activity and those involved in the cell cycle has been reported in ovarian cancer [38,39]. Recently, several genetic variations have been identified in genome-wide association studies (GWAS), and were found to act as low to moderate penetrant alleles, which contribute to ovarian cancer risk, as well as other diseases [4,40].

There is now an association of specific SNPs in key oxidant and anti-oxidant enzymes that impact increased risk of ovarian cancer and/or overall survival of patients with ovarian cancer [34,35]. A common SNP that reduced CAT activity (rs1001179) was utilized as a significant predictor of death when present in ovarian cancer patients and was also associated with increased risk for breast cancer [34,35,37,41]. This SNP is also linked to increased risk, survival, and response to adjuvant treatment of cancer patients, including ovarian [34,42]. Another common SNP that reduced CYBA activity (rs4673) was also reported to be associated with an increased risk for ovarian cancer [34,35]. The mutant genotype of the *CYBA* gene has been shown to both decrease and increase activity of the protein, thereby altering the generation of $O_2^{\bullet-}$ [34,35]. Moreover, functionally distinct *MPO* polymorphisms, such as (rs2333227) have been linked to relative increased risk for development of ovarian cancer as well as other cancers [34,35,43]. Additional SNPs that influenced the risk of EOC have been successfully identified from the GWAS studies including rs3814113 (located at 9p22, near BNC2), rs2072590 (located at 2q31, which contains a family of HOX genes), rs2665390 (located at 3q25, intronic to TIPARP), rs10088218 (located at 8q24, 700 kb downstream of MYC), rs8170 (located at 19p13, near MERIT40), and rs9303542 (located at 17q21, intronic to SKAP1)

[34,35]. Thus, the genetic component of increased ovarian cancer risk may be attributed to SNPs that result in point mutations in the redox genes and potentially other genes [44].

Chemoresistance is Associated with Point Mutations in Key Redox Enzymes in EOC cells

To date, the acquisition of chemoresistance in ovarian cancer is being investigated. The enhanced oxidant state reported in chemoresistant EOC cells is likely linked to point mutations in key redox enzymes [35]. Chemoresistant EOC cells manifested increased levels of CAT, GPX, and iNOS and decreased levels of GSR, SOD, and NAD(P)H oxidase as compared to their sensitive counterparts [35]. Interestingly, chemoresistant EOC cells, and not their sensitive counterparts, manifested specific point mutations that corresponded to known functional SNPs, in key redox enzymes including *SOD2* (rs4880), *NOS2* (rs2297518), and *CYBA* (rs4673) [45]. However, altered enzymatic activity for CAT and GSR observed in chemoresistant EOC cells did not correspond to the specific SNP of interest in those enzymes, indicating involvement of other possible functional SNPs for those enzymes [35]. Coincidently, chemotherapy treatment induced point mutations that happen to correspond to known functional SNPs in key oxidant enzymes subsequently led to the acquisition of chemoresistance by EOC cells. Indeed, the induction of specific point mutations in *SOD2* or *GPX1* in sensitive EOC cells resulted in a decrease in the sensitivity to chemotherapy of these cells [35]. In fact, the addition of *SOD* to sensitive EOC cells during chemotherapy treatment synergistically increased the efficacy to chemotherapy [35].

Alternatively, the observed nucleotide switch in response to chemotherapy in EOC cells may be the result of nucleotide substitution, a process that includes transitions, replacement of one purine by the other or that of one pyrimidine by the other, or transversions, replacement of a purine by a pyrimidine or vice versa [35]. Actually, hydroxyl radicals are known to react with DNA causing the formation of many pyrimidine and purine-derived lesions [35]. The oxidative damage to 8-Oxo-

2'-deoxyguanosine, a major product of DNA oxidation, induces genetic alterations in oncogenes and tumor suppressor genes that have been involved in tumor initiation and progression [35]. A GC to TA transversion has been reported in the *ras* oncogene and the *p53* tumor suppressor gene in several cancers. However, the GC to TA transversion is not unique to hydroxy-2'-deoxyguanosine, as CC to TT substitutions have been identified as signature mutations for oxidants and free radicals [35]. Moreover, the observed nucleotide switch in response to chemotherapy in EOC cells can be due to the fact that acquisition of chemoresistance generates an entirely different population of cells with a distinct genotype. Hence, chemotherapy kills the bulk of the tumor cells leaving a subtype of cancer cells with ability for repair and renewal, known as cancer stem cells (CSCs) [35]. Indeed, cancer stem cells have been isolated from various types of cancer including leukemia, breast, brain, pancreatic, prostate, ovary and colon [35]. Interestingly, CSC populations were present in cultures of SKOV-3 EOC cells and have been shown to be chemoresistant in nature [35].

Talcum powder and increased risk of ovarian cancer

Talcum powder is made from talc, a mineral containing mainly of the elements magnesium, silicon, and oxygen. In its natural form, some talc contains asbestos. Talc and asbestos are both silicate minerals; the carcinogenic effects of asbestos have been extensively studied and documented in the medical literature [46,47]. Asbestos fibers in the lung initiate an inflammatory and scarring process, and it has been proposed that ground talc, as a foreign body, initiates a similar inflammatory response and it has been proposed that ground talc, as a foreign body, might initiate an inflammatory response [48,47]. There has been concern about a possible link between talcum powder usage in the genital and ovarian cancer, as well as lung cancer in workers exposed to talc in an occupational setting [49]. Studies that exposed lab animals (rats, mice, and hamsters) to asbestos-free talcum powder in various ways have had mixed results, with some showing tumor

formation and others finding only inflammation [50,51]. The International Agency for Research on Cancer (IARC) is part of the World Health Organization (WHO). Its major goal is to identify causes of cancer. Based on limited evidence from human studies of a link to ovarian cancer, IARC classified the perineal (genital) use of talc-based body powder (not containing asbestiform fibers) as "possibly carcinogenic to humans." (Group 2b) [88]. Talcum powder containing asbestos and fibrous talc are both considered carcinogenic (Group 1) by IARC [89].

The association between perineal talc powder dusting and ovarian cancer has been studied in at least 25 case-control studies, three cohort studies, six meta-analyses and one pooled study [73]. Although the cohort studies individually did not show a statistically significant increased risk of ovarian cancer with talcum powder usage, the case-control studies overall and the meta-analyses show a consistent and significant increased risk. This risk is estimated to be 30-40%. The studies have shown conflicting results regarding the presence of a dose-response, largely due to the failure of many studies to obtain necessary information on the frequency and duration of usage and the inherent challenge of quantifying actual exposure. Although migration/transport of particles through the genital tract is universally accepted and the inflammatory nature of talcum powder consistently demonstrated, the exact mechanism for carcinogenesis had not been conclusively elucidated. For these reasons, there has been some reluctance in the scientific community to accept talcum powder as a causative risk factor for the development of ovarian cancer. The most recent meta-analysis, reported by Penninkilampi and Eslick in 2017, found that any perineal talc use was associated with a statistically significant increased risk of ovarian cancer (OR = 1.31). More than 3600 lifetime applications (OR = 1.42) were slightly more associated with ovarian cancer than <3600 (OR = 1.32). An association with ever use of talc was found in case–control studies (OR = 1.35), but not cohort studies (OR = 1.06). However, cohort studies found an association between

talc use and invasive serous type ovarian cancer (OR = 1.25), the most common and most lethal subtype. In the opinion of the authors, meta-analysis is the highest level of evidence in this setting because of the need for a large number of cases and long-term follow-up in a relatively rare form of cancer with a lengthy latency period. The authors of this meta-analysis suggested that cellular injury, oxidative stress, and local increase in inflammatory mediators might be the mechanism by which talcum powder promotes carcinogensis, but that this mechanism was unclear. They recognized the "substantial need for further research on a potential mechanism by which ovarian cancer may be caused by talc, as this will allow a causal relationship to be established or rejected with more certainty" [73].

In addition to epidemiological studies, the claim that regular use of talcum powder for perineal hygiene purpose is associated with an increased risk of ovarian cancer is based on several reports confirming the presence of talc particles in the ovaries and other parts of the female reproductive tract as well as in lymphatic vessels and tissues of the pelvis [45,75]. Henderson first reported the presence of talc particles in ovaries in 1971. A study by Cramer, et al has reported the presence of talc in pelvic lymph nodes of a woman with ovarian cancer who used talc daily for 30 years[45]. The ability of talc particles to migrate through the genital tract to the distal fallopian tube and ovaries is well accepted [45,75].

It has been suggested that the associations between perineal talc dusting and ovarian cancer might be explained by the induction of ANTI-MUC1 antibodies [57]. Additionally, in a previous study by Shukla et.al., whereby human mesothelial cells (LP9/TERT-1) were exposed to low and high (15 and 75 mm2/cm2 dish) equal surface area concentrations of nonfibrous talc for 8 or 24 hours, the authors found that nonfibrous talc at low concentrations to cause an increase in the

expression of Activating Transcription Factor 3 (ATF3) at 8 hours and no changes at 24 hours, whereas expression levels of 30 genes were elevated at 8 hours at high talc concentrations [78].

My laboratory undertook research to determine whether or not there was a molecular basis for the observed association between talcum powder and ovarian cancer. If a biological effect was demonstrated, we hoped to define the mechanism in detail. Issues like this one, relating to the pathogenesis of ovarian cancer and the relationship between inflammation and other pathological conditions in the female reproductive system as well as cancer, have been the focus of my laboratory for many years.

Findings from recent research from our laboratory relating to the effects of talcum powder exposure *in vitro*

The following is a description of the methodology used:

Cell Lines: Ovarian cancer cells: SKOV-3 (ATCC), A2780 (Sigma Aldrich), and TOV112D (a kind gift from Gen Sheng Wu at Wayne State University, Detroit, MI) [25]. Normal cell lines: human macrophage cells (EL-1, ATCC), human primary normal ovarian epithelial cells (Cell Biologics), Human Ovarian Epithelial Cells (HOSEpiC, ScienCell Research Laboratories, Inc.) immortalized human fallopian tube secretory epithelial cells (FT33-shp53-R24C, Applied Biological Materials). All cells were grown in media and conditions following manufacturer's protocol. EL-1 cells were grown in IMDM media (ATCC) supplemented with 0.1 mM hypoxanthine and 0.1 mM thymidine solution (H-T, ATCC) and 0.05mM β-mercaptoethanol. SKOV-3 EOC cells were grown in HyClone McCoy's 5A medium (Fisher Scientific), A2780 EOC cells were grown in HyClone RPMI-1640 (Fisher Scientific), and both TOV112D EOC cells were grown in MCDB105 (Cell Applications) and Medium 199 (Fisher Scientific) (1:1). All media was supplemented with fetal bovine serum (FBS, Innovative Research) and penicillin/streptomycin

(Fisher Scientific), per their manufacturer specifications. Human primary normal ovarian epithelial cells were grown in Complete Human Epithelial Cell Medium (Cell Biologics).

Treatment of cells: Talcum powder (Fisher Scientific, Catalog #T4-500, Lot#166820) or Johnson's Baby Powder (Johnson & Johnson, #30027477, Lot#13717RA) was dissolved in DMSO (Sigma Aldrich) at a concentration of 500 mg in 10 ml and was filtered with a 0.2 µm syringe filter (Corning). Sterile DMSO was used as a control for all treatments. Cells were seeded in 100 mm cell culture dishes (3×10^6) and were treated 24 hours later with 0, 5, 20, or 100 µg/ml of talc for 48 hours. Cell pellets were collected for RNA, DNA, and protein extraction. Cell culture media was collected for CA-125 analysis by ELISA.

Real time RT-PCR: Total RNA was extracted from all cells using the RNeasy Mini Kit (Qiagen) according to the protocol provided by the manufacturer. Measurement of the amount of RNA in each sample was performed using a Nanodrop Spectrophotometer (Thermo Fisher Scientific). A 20 µL cDNA reaction volume containing 0.5 µg RNA was prepared using the SuperScript VILO Master Mix Kit (Life Technologies), as described by the manufacturer's protocol. Optimal oligonucleotide primer pairs were selected for each target using Beacon Designer (Premier Biosoft, Inc., Table 1). Quantitative RT-PCR was performed using the EXPRESS SYBR Green ER qPCR Supermix Kit (Life Technologies) and the Cepheid 1.2f Detection System as previously described[24]. Standards with known concentrations and lengths were designed specifically for β-actin (79 bp), CAT (105 bp), iNOS (89 bp), GSR (103 bp), GPX1 (100 bp), MPO (79 bp), and SOD3 (84 bp), allowing for construction of a standard curve using a 10-fold dilution series[26]. A specific standard for each gene allows for absolute quantification of the gene in number of copies, which can then be expressed per microgram of RNA. All samples

were normalized to the housekeeping gene, β-actin. A final melting curve analysis was performed to demonstrate specificity of the PCR product.

Protein Detection: Cell pellets were lysed utilizing cell lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na3VO4, 1 µg/ml leupeptin) containing a cocktail of protease inhibitors. Samples were centrifuged at 13000 rpm for 10 minutes at 4°C. Total protein concentration of cell lysates from control and talc-treated cells was measured with the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, Illinois) per the manufacturer's protocol. *Detection of protein/activity by ELISA:* ELISA kits for each target were purchased and used according to the manufacturer's protocol. The following ELISA kits were purchased from Cayman Chemical, Ann Arbor, MI: CAT, SOD3, GSR, GPX1, and MPO. Nitrite ($NO2^-$)/nitrate ($NO3^-$) were determined spectrophotometrically by measuring their absorbance at 210 nm after separation by HPLC with standard $NO2^-$/$NO3^-$ as previously reported[19]. The analysis was performed by a HPLC system (Shimadzu Scientific Instruments, Inc.) including a LC-10ADV pump, frc-10A injector and DGU-14A degasser. Nitrite/nitrate were detected using an RF-10 XL fluorescence detector with 210 nm excitation and 440 nm emission. CA-125 protein levels were measured in cell media by ELISA from Ray Biotech according to the manufacturer's protocol.

TaqMan® SNP Genotyping Assay: DNA was isolated utilizing the EZ1 DNA Tissue Kit (Qiagen Valencia, CA) for EOC cells according the manufacturer's protocols. The TaqMan® SNP Genotyping Assay Set (Applied Biosystems, Carlsbad, CA) (NCBI dbSNP genome build 37, MAF source 1000 genomes) were used to genotype the SNPs described in Table 1. The Applied Genomics Technology Center (AGTC, Wayne State University, Detroit, MI) performed these

assays. Analysis was done utilizing the QuantStudioTM 12 K Flex Real-Time PCR System (Applied Biosystems).

Statistical Analysis: Normality was examined using the Kolmogorov-Smirnov test and by visual inspection of quantile-quantile plots. Because most of the data were not normally distributed, differences in distributions were examined using the Kruskal-Wallis test. Generalized linear models were fit to examine pairwise differences in estimated least squares mean expression values by exposure to 0, 5, 20 or 100 ug/ml of Talc. We used the Tukey-Kramer adjustment for multiple comparisons, and the regression models were fit using log2 transformed analyte expression values after adding a numeric constant '1' to meet model assumptions while avoiding negative transformed values. P-values below 0.05 are statistically significant.

Research Findings: Recent studies from our laboratory have shown conclusively that talcum powder alter key redox and inflammatory markers, enhance cell proliferation, and inhibit apoptosis in EOC cells, which are hallmarks of ovarian cancer. More importantly, this effect is also manifested by talcum powder in normal cells, including surface ovarian epithelium, fallopian tube, and macrophages. Oxidative stress has been implicated in the pathogenesis of ovarian cancer, specifically, by increased expression of several key pro-oxidant enzymes such as iNOS, MPO, and NAD(P)H oxidase in EOC tissues and cells as compared to normal cells indicating an enhanced redox state, as we have recently demonstrated [19]. This redox state is further enhanced in chemoresistant EOC cells as evident by a further increase in iNOS and $NO2^-/NO3^-$ and a decrease in GSR levels, suggesting a shift towards a pro-oxidant state [19]. Antioxidant enzymes, key regulators of cellular redox balance, are differentially expressed in various cancers, including ovarian [19,79]. Specifically, GPX expression is reduced in prostate, bladder, and estrogen receptor negative breast cancer cell lines as well as in cancerous tissues from the kidney. However, GPX

activity is increased in cancerous tissues from breast [79]. Glutathione reductase levels, on the other hand, are elevated in lung cancer, although differentially expressed in breast and kidney cancerous tissues [5,80]. Similarly, CAT was decreased in breast, bladder, and lung cancer while increased in brain cancer [81-83]. Superoxide dismutase is expressed in lung, colorectal, gastric ovarian, and breast cancer, while decreased activity and expression have been reported in colorectal carcinomas and pancreatic cancer cells [83-86]. Collectively, this differential expression of antioxidants demonstrates the unique and complex redox microenvironment in cancer. Glutathione reductase is a flavoprotein that catalyzes the NADPH-dependent reduction of oxidized glutathione (GSSG) to GSH. This enzyme is essential for the GSH redox cycle which maintains adequate levels of reduced cellular GSH. A high GSH/GSSG ratio is essential for protection against oxidative stress. Treatment with talc significantly reduced GSR in normal and cancer cells, altering the redox balance. Likewise, GPX is an enzyme that detoxifies reactive electrophilic intermediates and thus plays an important role in protecting cells from cytotoxic and carcinogenic agents. Overexpression of GPX is triggered by exogenous chemical agents and reactive oxygen species, and is thus thought to represent an adaptive response to stress [80]. Treatment of normal and cancer cells with talc significantly reduced GPX, which compromised the overall cell response to stress.

We have previously reported that EOC cells manifest increased cell proliferations and decreased apoptosis [19]. Consistent with these findings, recent studies from my laboratory have shown that talc enhances cell proliferation and induces an inhibition in apoptosis in EOC cells, but more importantly in normal cells, suggesting talc is a stimulus to the development of the oncogenic phenotype. We also previously reported a cross-talk between iNOS and MPO in ovarian cancer which contributed to the lower apoptosis observed in ovarian cancer cells [17,19]. Collectively, we now have substantial evidence demonstrating that altered oxidative stress may play a role in

maintaining the oncogenic phenotype of EOC cells. Treatment of normal or ovarian cancer cells with talc resulted in a significant increase in MPO and iNOS, supporting the role of talc in the enhancement of a pro-oxidant state that is a major cause in the development and maintenance of the oncogenic phenotype.

Furthermore, CA-125, which exists as a membrane-bound and secreted protein in epithelial ovarian cancer cells, has been established as a biomarker for disease progression and response to treatment [2]. CA-125 expression was significantly increased from nearly undetectable levels in controls to values approaching clinical significance (35 U/ml in postmenopausal women[87]) in talc treated cell lines without the physiologic effects on the tumor microenvironment one would expect to be present in the human body, highlighting the implications of the pro-oxidant states caused by talc alone.

To elucidate the mechanism by which talc alters the redox balance to favor a pro-oxidant state not only in ovarian cancer cells, but more importantly in normal cells, my laboratory examined selected known gene mutations in key oxidant and antioxidant enzymes. These mutations correspond to specific SNPs that are known to be associated with altered enzymatic activity and increased cancer risk [34,35]. Results show that the *CAT* SNP (rs769217) which results in decreased enzymatic activity was induced in all normal cell lines tested and in TOV112D EOC lines. However, the *CAT* mutation was not detected in A2780 or SKOV-3 cell lines. Nevertheless, our results confirm a decrease in CAT expression and enzymatic activity in all talc treated cells, indicating the existence of other *CAT* SNPs. However, the *SOD3* (rs2536512) and *GSR* (rs8190955) SNP genotypes were not detected in any cell line, yet SOD3 and GSR activity and expression were decreased in all talc treated cells, again suggesting the presence of other SNPs. Our results have also shown that all cells, except for HOSEpiC cells, manifest the SNP genotype

of *GPX1* (C/T) before talc treatment. Intriguingly, talc treatment reversed this SNP genotype to the normal genotype. Consistent with this finding, it has previously been reported that acquisition of chemoresistance by ovarian cancer cells is associated with a switch from the *GPX1* SNP genotype to the normal *GPX1* genotype [35]. It is not understood why a *GPX1* SNP genotype predominates in untreated normal and ovarian cancer cells. Additionally, our results showed that talc treatment was associated with a genotype switch from common C/C genotype in *NOS2* in untreated cells to T/T, the SNP genotype, in talc treated cells, except in A2780 and TOV112D. Nevertheless, our results confirm an increase in iNOS expression and enzymatic activity in all talc treated cells, again suggesting the existence of other *NOS2* SNPs. Collectively, these findings demonstrate that talc treatment induced gene point mutations that happen to correspond to SNPs in locations with functional effects, thus altering overall redox balance for the initiation and development of ovarian cancer. Future studies examining such SNPs are important to fully elucidate a genotype switch mechanism induced by talc exposure.

In summary, this research clearly demonstrates that talcum powder induces inflammation and alters the redox balance favoring a pro-oxidant state in normal and EOC cells. This study has shown a dose-dependent significant increase in key pro-oxidants, iNOS, $NO2^-/NO3^-$, and MPO and a concomitant decrease in key antioxidant enzymes, CAT, SOD, GPX, and GSR, in all talc treated cells (both normal and ovarian cancer) compared to their controls. Additionally, there was a significant increase in CA-125 levels in all the talc treated cells compared to their controls, except in macrophages (which do not produce CA-125). The mechanism by which talc alters the cellular redox and inflammatory balance involves the induction of specific mutations in key oxidant and antioxidant enzymes that correlate with alterations in their activities. The fact that these mutations

happen to correspond to known SNPs of these enzymes indicate a genetic predisposition to developing ovarian cancer with genital talcum powder exposure.

Summary of opinions

These opinions are made to a reasonable degree of scientific certainty and are based on my experience, training, and expertise, as well as a knowledge of the relevant scientific literature and my previous and ongoing research.

1. Johnson's Baby Powder elicits an inflammatory response in normal ovarian and tubal cells and in ovarian cancer cells that can result in the development and the progression of ovarian cancer.
2. This pro-carcinogenic process involves oxidative stress, alteration of the redox environment by increasing oxidant enzymes and decreasing anti-oxidant enzymes, promotion of cell proliferation, inhibition of apoptosis, and induction of specific genetic mutations.
3. Johnson's Baby Powder exposure results in elevation of CA-125, a clinically relevant biomarker for ovarian cancer, in normal and ovarian cancer cells.
4. The molecular effects resulting from Johnson's Baby Powder exposure exhibit a clear dose-response pattern.
5. In my opinion, based on established molecular mechanisms for the pathogenesis of ovarian cancer (as evidenced in the peer-reviewed scientific literature and my previously published research) and my *in vitro* experiments, Johnson's Baby Powder exposure can cause ovarian cancer.
6. In my opinion, based on established molecular mechanisms that influence the progression and chemoresistance associated with ovarian cancer (as evidenced in the peer-reviewed

scientific literature and my previously published research) and my *in vitro* experiments, Johnson's Baby Powder exposure worsens the prognosis for patients with ovarian cancer.

I reserve the right to amend or supplement this report as new information becomes available.

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Exhibit 106

DOI:10.1093/jnci/djt431

ARTICLE

Aspirin, Nonaspirin Nonsteroidal Anti-inflammatory Drug, and Acetaminophen Use and Risk of Invasive Epithelial Ovarian Cancer: A Pooled Analysis in the Ovarian Cancer Association Consortium

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Manuscript received April 10, 2013; revised November 10, 2013; accepted November 14, 2013.

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Background Regular aspirin use is associated with reduced risk of several malignancies. Epidemiologic studies analyzing aspirin, nonaspirin nonsteroidal anti-inflammatory drug (NSAID), and acetaminophen use and ovarian cancer risk have been inconclusive.

Methods We analyzed pooled data from 12 population-based case–control studies of ovarian cancer, including 7776 case patients and 11 843 control subjects accrued between 1992 and 2007. Odds ratios (ORs) for associations of medication use with invasive epithelial ovarian cancer were estimated in individual studies using logistic regression and combined using random effects meta-analysis. Associations between frequency, dose, and duration of analgesic use and risk of ovarian cancer were also assessed. All statistical tests were two-sided.

Results Aspirin use was associated with a reduced risk of ovarian cancer (OR = 0.91; 95% confidence interval [CI] = 0.84 to 0.99). Results were similar but not statistically significant for nonaspirin NSAIDs, and there was no association with acetaminophen. In seven studies with frequency data, the reduced risk was strongest among daily aspirin users (OR = 0.80; 95% CI = 0.67 to 0.96). In three studies with dose information, the reduced risk was strongest among users of low dose (<100 mg) aspirin (OR = 0.66; 95% CI = 0.53 to 0.83), whereas for nonaspirin NSAIDs, the reduced risk was strongest for high dose (≥500 mg) usage (OR = 0.76; 95% CI = 0.64 to 0.91).

Conclusions Aspirin use was associated with a reduced risk of ovarian cancer, especially among daily users of low-dose aspirin. These findings suggest that the same aspirin regimen proven to protect against cardiovascular events and several cancers could reduce the risk of ovarian cancer 20% to 34% depending on frequency and dose of use.

JNCI J Natl Cancer Inst (2014) 106(2): djt431 doi:10.1093/jnci/djt431

Ovarian cancer is the most fatal gynecologic malignancy, causing more than 140 000 deaths each year worldwide (1). Although early stage ovarian cancer can be successfully treated, the disease is commonly detected at advanced stages with extensive local and systemic spread and poor survival. Early detection strategies have not been shown to reduce mortality (2,3), and biomarker candidates have had insufficient performance to improve early detection efforts thus far (4,5). Primary prevention strategies have not been widely studied but may present alternatives to reduce ovarian cancer burden.

Multiple lines of evidence suggest that ovarian cancer may be related to chronic inflammation (6). In addition to inflammatory factors associated with ovarian epithelial disruption through ovulation (7–9), inflammation-related exposures such as endometriosis (10–12) and exposure to talc or genital powder and asbestos (13) have been associated with increased ovarian cancer risk.

Recently, intervention trials have shown that regular aspirin use is associated with reduced risk of several malignancies (14). However, these trials were not powered for rare cancer endpoints, and none of the clinical trials to date have evaluated ovarian cancer separately. Recent meta-analyses of aspirin use have reached various conclusions that range from no effect (15) to a weak risk reduction among regular users of aspirin (16–18). For nonsteroidal

anti-inflammatory drug (NSAID) use, a recent summary suggested a greater risk reduction among cohort studies than among case–control studies (15), whereas, the results from individual epidemiologic studies have been largely inconclusive (13,19–33), possibly because of limited sample size and limited data on dose, duration, and frequency of use across the studies.

We conducted an analysis of pooled individual-level data of NSAID use and ovarian cancer risk in the Ovarian Cancer Association Consortium (OCAC), including more than 7500 ovarian cancer cases from 12 population-based case–control studies.

Methods

Study Population

We analyzed individual-level data from 12 population-based case–control studies participating in OCAC that had available data on aspirin, nonaspirin NSAID, or acetaminophen (paracetamol) use. All studies had approval from ethics committees, and written informed consent was obtained from study participants. Data acquisition and data pooling for each study were approved by the institutional review board or research ethics committees of the institutes sponsoring the study.

The 12 studies were as follows: the Australian Ovarian Cancer Study and Australian Cancer Study (26), the Connecticut Ovarian Cancer Study (34), the Diseases of the Ovary and their Evaluation Study (23,35), the Hawaii Ovarian Cancer Case–Control Study (36,37), the Hormones and Ovarian Cancer Prediction Study (38), the Malignant Ovarian Cancer Study (39), the North Carolina Ovarian Cancer Study (40,41), the New England Case–Control Study of Ovarian Cancer (42), the New Jersey Ovarian Cancer Study (43), the University of California, Irvine Ovarian Cancer Study (44), the United Kingdom Ovarian Cancer Population Study (45), and the University of Southern California Study of Lifestyle and Women's Health (13) (Table 1). In total, the study included data from nine case–control studies conducted in the United States (13,23,34,37,38,40,42–44), one study conducted in Denmark (39), one study conducted in the United Kingdom (45), and one study conducted in Australia (26).

From these 12 studies, 10 161 ovarian cancer case patients and 12 382 control subjects were available for the analysis. For the primary analysis, we excluded case patients whose cancers were nonepithelial (n = 43), of low malignant potential (n = 2059), or missing data on both the malignant potential of the tumor and tumor grade (n = 68). We further excluded study participants with missing data for all three exposure variables (n = 215 case patients and n=539 control subjects), leaving 7776 invasive ovarian cancer case patients and 11 843 control subjects for our analysis. The case patients were divided into four categories by the four main histologic subtypes of the cancer: serous (n = 4510), endometrioid (n = 1163), clear cell (n = 677), and mucinous (n = 423). The remaining 1003 case patients with cancers of other histologic type were not included in subtype analyses. We also evaluated associations for high-grade serous ovarian tumors (grade II–IV; n = 3786) based on the prevailing view that high-grade serous tumors are distinct from low-grade (grade I; n = 330) serous tumors (46). We evaluated 2059 case patients with cancers of low malignant potential in a separate analysis.

Study Variables

Data for medication use was self-reported in all studies (Table 1). Ten of the 12 studies asked about "regular use" of medications over a specified time period with a minimum frequency of use (13,23,34,38–40,42–45). The duration of regular use varied in the 10 studies, from 1 month to 1 year of use. The majority of the studies, six of 10, specified 6 months or more as the minimum duration (23,38,42–45). The definition for frequency of regular use also varied by study, ranging from once per week to daily; the majority of the studies (n = 8 of 10) specified once or twice per week as the minimum frequency of regular use (13,23,34,38,39,42,44,45). The two remaining studies did not specify regular use, so we reclassified study participants as regular users if their reported frequency of use was at least once per week (26) or if their frequency of use was at least five pills per month and their duration of use was at least 6 months (37).

The exposures used in this analysis were regular (at least once per week) use of aspirin, nonaspirin NSAIDs, and acetaminophen and nonregular use (reference group; less than once a week use for each category). Data for nonaspirin NSAID use were provided in all studies except for two studies that combined aspirin use with other NSAIDs (44,45). Medication use was further classified by frequency [<30 days per month and daily; n = 7 studies (13,23,26,37-40)], dose [<100 and ≥100 mg for aspirin to differentiate between use of low- and regular/high-dose formulations; <500 mg and ≥ 500 mg for non-aspirin NSAID and acetaminophen to differentiate between standard and high-dose formulations; n = studies (37,38,40)], and duration [<60 months and ≥60 months; n = 8 studies (13,23,34,37-39,42,43)] of use based on available data from the individual studies. We created a frequency–dose combination exposure variable based on cross-tabulations of the original categorical variables [(n = 3 studies) (37,38,40)].

Potential confounding variables were available from all studies as part of a core dataset and were harmonized by the coordinating center. Continuous variables were categorized in all analyses for ease of interpretation and to reduce the effect of any outliers. Variables that were selected a priori as adjustment factors included age (5-year categories), race (white, black, other), body mass index (<25, 25–29, ≥30 kg/m^2), use of oral contraceptives (ever, never), parity (nulliparous, 1 full-term birth, >1 full-term birth), menopausal status (pre- or postmenopausal based on study-specific algorithm), and family history of breast or ovarian cancer in a first-degree relative (defined as any breast or ovarian cancer reported in mother, sister, or daughter or breast cancer reported in father). Potential confounding was also evaluated, but not found, for the following variables: Hispanic ethnicity, history of breast feeding, use of estrogen menopausal hormone therapy, use of estrogen plus progestin menopausal hormone therapy, tubal ligation, hysterectomy, and history of endometriosis.

Statistical Analyses

We used multivariable logistic regression models to estimate study-specific odds ratios (ORs) and 95% confidence intervals (CIs) for the association between NSAID exposure and ovarian cancer risk. Study-specific odds ratios were pooled using random-effects meta-analysis to generate a summary odds ratio. For the analyses of the primary exposures (regular use, dose, duration, and frequency), two

Table 1. Characteristics of population-based case–control studies from the Ovarian Cancer Association Consortium included in the pooled analysis*

Study	OCAC acronym	Location	Ascertainment period	Study subjects: Case patients (n = 7776)	Study subjects: Control subjects (n = 11843)	Question pertaining to drug use	Prevalence of exposure in control subjects: Aspirin %	Nonaspirin NSAID‡ %	Acetaminophen %
Australian Ovarian Cancer Study & Australian Cancer Study† (26)	AUS	Australia	2002–2005	1311	1505	How often have you taken the following over-the-counter (aspirin, paracetamol, anti-inflammatory drugs) medications during PAST 5 years?	10	16	25
Connecticut Ovarian Cancer Study (34)	CON	USA	1999–2003	388	551	Have you ever taken any of the medications shown on this card regularly (at least once per week on average over a duration of 3 months or more)?	26	28	16
Diseases of the Ovary and their Evaluation Study (23,35)	DOV	USA	2002–2009	1159	1849	Before reference date have you taken any of these medications (show card) 5 or more days per month for at least 6 months?	22	27	16
Hawaii Ovarian Cancer Case–Control Study (36,37)	HAW	USA	2001–2008	256	485	Did you ever take an aspirin product (show card) at least 12 times a year? Identical questions ascertained use of acetaminophen (aspirin-free) and NSAIDs.	26	25	22
Hormones and Ovarian Cancer Prediction Study (38)	HOP	USA	2003–2008	683	1513	Prior to reference date have you ever used aspirin (show card) for at least two tablets per week continuously for a period of 6 months or longer? Identical questions ascertained use of over-the-counter pain or inflammation reliever other than aspirin.	34	33	19
Malignant Ovarian Cancer Study (39)	MAL	Denmark	1994–1999	554	1564	Did you ever take medicine on a regular basis, i.e. two times or more per week for more than one month for any of the following conditions?	8	9	5
North Carolina Ovarian Cancer Study (40,41)	NCO	USA	1999–2008	939	1085	For the 5 years prior to diagnosis, did you take any of these over-the-counter medications (show card) on a regular basis for at least 3 months?	11	38	20
New England Case–Control Study of Ovarian Cancer (42)	NEC	USA	1992–2003	870	1243	Prior to reference date have you ever used any over-the-counter pain reliever (show card) continuously at least once a week for a period of 6 months or longer?	18	25	22

(Table continues)

Table 1 (*Continued*).

Study	OCAC acronym	Location	Ascertainment period	Study subjects: Case patients (n = 7776)	Study subjects: Control subjects (n = 11843)	Question pertaining to drug use	Prevalence of exposure in control subjects: Aspirin %	Prevalence of exposure in control subjects: Nonaspirin NSAID‡ %	Prevalence of exposure in control subjects: Acetaminophen %
New Jersey Ovarian Cancer Study (43)	NJO	USA	2002–2008	238	458	Prior to reference date did you ever take any over-the-counter medications continuously for 6 months or longer (this includes prescriptions, over-the-counter medications, and any natural or alternative treatments you may have taken).	16	9	3
University of California, Irvine Ovarian Cancer Study (44)	UCI	USA	1995–2005	393	313	Have you taken medication listed (aspirin, ibuprofen, acetaminophen, naproxen) regularly? By regular, we are referring to use of the drug or medication at least once a week for a year, or more than 50 pills during a one year-period.	26	41‡	17
United Kingdom Ovarian Cancer Population Study (45)	UKO	UK	2006–2007	516	598	Have you ever used any medication containing the drugs (aspirin, ibuprofen) on a regular basis (by regular we mean every day or almost every day for 6 months or longer)?	15	16‡	—
University of Southern California Study of Lifestyle and Women's Health (13)	USC	USA	2000–2005	469	679	Before reference date, as an adult, did you ever take any prescription or non-prescription medicine at least 2 or more times per week for one month or longer?	15	16	13
						Overall	18	24	16

* NSAID = nonsteroidal antiinflammatory drug; OCAC = Ovarian Cancer Association Consortium.

† Combined for the purpose of this analysis.

‡ UCI and UKO reported data on NSAIDs, including aspirin; the remaining studies provided data on nonaspirin NSAIDs.

multivariable logistic regression models were used: 1) a minimally adjusted model that included covariables for age and race and 2) a fully adjusted model that included age, race, body mass index, oral contraceptive use, parity, menopausal status, and family history of breast or ovarian cancer in a first-degree relative. The summary odds ratios from the fully adjusted model were attenuated slightly compared with the minimally adjusted model. We present the results from the fully adjusted model. We further evaluated models stratified by age (<55 and ≥55 years old), body mass index (<25 and ≥25 kg/m^2), oral contraceptive use (ever/never), and history of endometriosis (yes/no). We assessed asymmetry in study estimates using a funnel plot, and when data were sufficient (n > 5 studies), we formally assessed asymmetry using the adjusted rank correlation (47) and regression asymmetry tests (48). Interstudy heterogeneity was evaluated using I^2.

The following sensitivity analyses were performed: 1) exclusion of tubal or primary peritoneal cases (n = 461); 2) restriction to white non-Hispanic participants because 85% of the participants were of white race and non-Hispanic ethnicity; 3) use of a common reference group analysis, coding "nonregular users" as women who reported no regular use of aspirin or nonaspirin NSAIDs or acetaminophen; 4) restriction of pooled analysis to the six studies that specified 6 months or more as the minimum duration; 5) restriction of pooled analysis to the nine US studies; and 6) exclusion from the pooled analysis the two studies (23,45) with the most restrictive definition of medication use given concerns for misclassification of regular users as unexposed. All statistical tests were two-sided, and P values less than .05 were considered statistically significant. All analyses were performed using STATA software version 11.2 (StataCorp LP, College Station, TX).

Results

Study site, number of case patients and control subjects, and exposure prevalence for each of the 12 OCAC studies are described in Table 1. Overall, 18% of the study population reported regular use (at least once per week) of aspirin, 24% reported regular use of nonaspirin NSAIDs, and 16% reported regular use of acetaminophen.

Aspirin

Figure 1A shows the association between aspirin use (regular vs nonregular use) and ovarian cancer risk. Regular aspirin use was associated with a reduced risk of ovarian cancer (OR = 0.91; 95% CI = 0.84 to 0.99; I^2 = 5.2%). Among seven studies that reported information on frequency of use, daily use was associated with a 20% reduction in ovarian cancer risk (OR = 0.80; 95% CI = 0.67 to 0.96) (Table 2). Among three studies that reported information on dose, low-dose aspirin use (<100 mg/day) was associated with a 34% reduction in ovarian cancer risk (OR = 0.66; 95% CI = 0.53 to 0.83) (Table 2). In analyses of combined categories of frequency and dose of aspirin use, the reduced risk was apparent for daily users of aspirin regardless of dose (low dose: OR = 0.64, 95% CI = 0.50 to 0.81; high dose: OR = 0.78, 95% CI = 0.62 to 0.97) (Table 3).

In subtype analyses, regular aspirin use was associated with reduced risks of serous, endometrioid, and mucinous ovarian cancer, but only the results for serous cancer reached statistical significance (OR = 0.89; 95% CI = 0.80 to 0.99) (Table 4). Pairwise comparisons showed no significant differences in risk between the subtypes (P > .05).

Nonaspirin NSAIDs

Regular nonaspirin NSAID use was associated with a reduced, albeit not statistically significant, risk of ovarian cancer (OR = 0.90; 95% CI = 0.77 to 1.05; I^2 = 73.2%) (Figure 1B). Among the three studies that reported information on dose, high-dose nonaspirin NSAID use (≥500 mg/day) was associated with a 24% reduction in ovarian cancer risk (OR = 0.76; 95% CI = 0.64 to 0.91) (Table 2). In analyses of combined categories of frequency and dose, the reduced risk of ovarian cancer was apparent among both categories of high-dose nonaspirin NSAID use (<30 days per month: OR = 0.77, 95% CI = 0.57 to 1.04; daily: OR = 0.75; 95% CI = 0.60 to 0.94), with a weaker association with daily users of low-dose nonaspirin NSAIDs (OR = 0.88; 95% CI = 0.70 to 1.11) (Table 3). The association between nonaspirin NSAIDs and risk was strongest for serous cancers but did not differ across histologic subtypes of ovarian cancer (Table 4).

Acetaminophen

Acetaminophen use was not associated with ovarian cancer risk (OR = 0.99; 95% CI = 0.88 to 1.12; I^2: 40.0%) (Figure 1C). No associations were observed when analyzing dose, duration, or frequency of acetaminophen use and ovarian cancer risk (Table 2). Further we observed no association between acetaminophen use and histologic subtypes of ovarian cancer (Table 4).

Additional Analyses

The association between NSAID use and high-grade serous tumors was not substantially different than the results reported for all serous tumors combined (results not shown). Tumors of low malignant potential (n = 2059) were not associated with analgesic use (data not shown). In analyses stratified by age, body mass index, oral contraception use, and history of endometriosis, similar NSAID use and ovarian cancer associations were observed as in the overall population (results not shown). Based on the adjusted rank correlation and regression asymmetry tests, there was no indication of small study effects (all P > .05) in the summary estimates for the associations between regular use of aspirin, nonaspirin NSAIDs, or acetaminophen and ovarian cancer. Although there was heterogeneity in the definition of nonaspirin NSAID use, individual exclusion of each study did not substantially change the summary odds ratio (results not shown); however, the exclusion of two studies (13,44) resulted in a decrease in I^2 from 73.2% to 27.8% but no substantial change in the summary odds ratio (results not shown).

In a sensitivity analysis excluding peritoneal and fallopian tube cancers, the pooled summary odds ratios for the associations between regular use of aspirin, nonaspirin NSAIDs, or acetaminophen and ovarian cancer were not substantially different from the odds ratios observed for the overall case group (data not shown). The associations between regular use of NSAIDs and ovarian cancer did not substantially change when the analyses were restricted to non-Hispanic white case patients and control subjects (data not shown). In analyses using women who reported nonregular use of all three NSAIDs as the reference group, a stronger reduced risk was observed for regular use of aspirin (OR = 0.81;

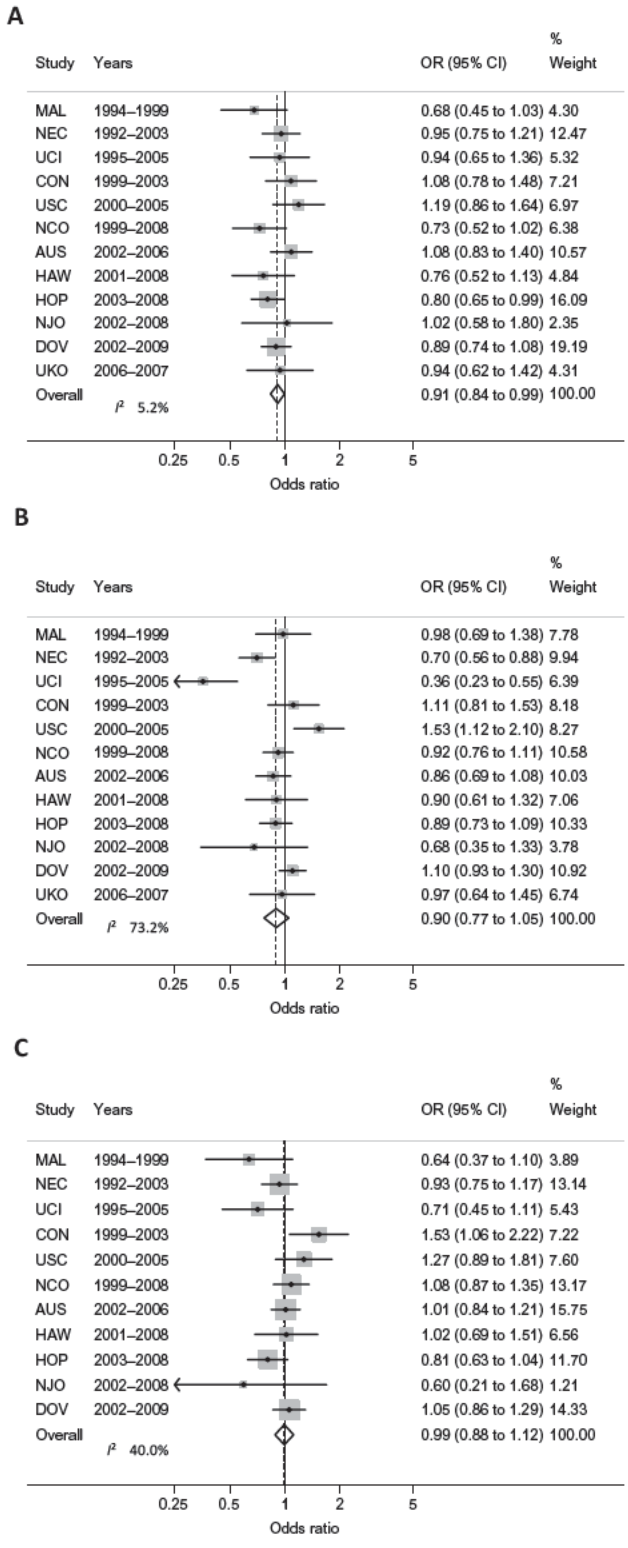

Figure 1. The summary odds ratios (ORs) and 95% confidence intervals (CIs) for the association between regular (at least once per week) use of aspirin (**A**), nonaspirin nonsteroidal anti-inflammatory drugs (NSAIDs) (**B**), and acetaminophen (**C**) and ovarian cancer risk. Summary odds ratios and 95% confidence intervals were estimated using a random-effect meta-analytic model. All statistical tests were two-sided. I^2 is the percentage of variation across studies due to heterogeneity rather than chance. % Weight describes the weight (inverse variance) each study contributed to the summary odds ratio, and the size of the surrounding **square** is an illustrative representation of study weighting. The **horizontal lines** represent study-specific confidence intervals; if ending in an **arrow**, this indicates that the interval transcends the region plotted. The **diamond** represents the summary odds ratio and 95% confidence interval. Studies are presented in order of median year of case accrual from earliest to most recent. AUS = Australian Ovarian Cancer Study, Australian Cancer Study; CON = Connecticut Ovary Study; DOV = Diseases of the Ovary and their Evaluation Study; HAW = Hawaii Ovarian Cancer Study; HOP = Hormones and Ovarian Cancer Prediction Study; MAL = Malignant Ovarian Cancer Study; NCO = North Carolina Ovarian Cancer Study; NEC = New England Case–Control Study of Ovarian Cancer; NJO = New Jersey Ovarian Cancer Study; UCI = University of California, Irvine Ovarian Cancer Study; UKO = United Kingdom Ovarian Cancer Population Study; USC = University of Southern California Study of Lifestyle and Women's Health.

95% CI = 0.68–0.99) and nonaspirin NSAID (OR = 0.86; 95% CI = 0.71–1.05), possibly reflecting reduced "contamination" of the referent group with users of NSAID types other than the medication under examination in each specific analysis (data not shown). In sensitivity analyses restricted to the six studies that specified 6 months or more as the minimum duration or the nine US studies, the pooled summary odds ratios for the associations between regular use of aspirin, nonaspirin NSAIDs, or acetaminophen and ovarian cancer were not substantially different from the odds ratios observed for the overall pooled analysis (data not shown). Finally, in the sensitivity analysis excluding case patients with the most restrictive definition of medication use, the pooled summary odds ratios for the associations between regular use of aspirin, nonaspirin NSAIDs, or acetaminophen and ovarian cancer were not substantially different from the pooled odds ratios observed for all 12 studies (data not shown).

Discussion

To our knowledge, this is the largest evaluation of aspirin, nonaspirin NSAID, and acetaminophen use and ovarian cancer risk to date. We observed a 20% risk reduction for daily users of aspirin and 34% risk reduction for regular users of low-dose aspirin. Regular (at least once per week) use of high doses of nonaspirin NSAIDs was associated with a 24% reduction in ovarian cancer risk. In contrast, acetaminophen use was not associated with ovarian cancer risk. We did not observe any substantial differences in risk by histologic subtypes of ovarian cancer.

Several established risk factors for ovarian cancer are related to inflammatory processes. During ovulation, follicles rupture and inflammatory mediators are released locally that may initiate cell transformation or that may promote growth of transformed cells (49). Proinflammatory agents are also released in inflammatory processes related to endometriosis (10). Aspirin and nonaspirin NSAIDs may reduce exposure to these inflammatory processes; thus, the reduced risk of ovarian cancer with frequent aspirin and nonaspirin NSAID use is consistent with the hypothesized inflammatory etiology of ovarian cancer (50). Several observational studies have evaluated NSAID use and the risk of ovarian cancer. (13,15,19–33,51) A recent meta-analysis reported comparable summary odds ratios for any use of aspirin (OR = 0.91; 95% CI = 0.82 to 1.01) and nonaspirin NSAIDs (OR = 0.89; 95% CI = 0.74 to 1.08), but the estimates did not reach statistical significance (51).

Table 2. Summary odds ratios (ORs) and 95% confidence intervals (CIs) for the associations of aspirin, nonaspirin NSAID, and acetaminophen/paracetamol use with risk of ovarian cancer in the Ovarian Cancer Association Consortium (1992–2009)*

Exposure categorization	Aspirin					Nonaspirin NSAID					Acetaminophen				
	Control	Case	OR†	(95% CI)	I^2‖	Control	Case	OR†	(95% CI)	I^2‖	Control	Case	OR†	(95% CI)	I^2‖
Frequency‡															
No regular use	6366	3826	1.00	(referent)		6007	3565	1.00	(referent)		6189	3497	1.00	(referent)	
<30 days per month	917	739	1.04	(0.92 to 1.18)	0.0	1357	994	1.04	(0.88 to 1.22)	44.8	1805	1439	1.10	(0.96 to 1.26)	0.0
Daily	1179	607	0.80	(0.67 to 0.96)	51.4	1285	776	0.97	(0.83 to 1.12)	46.1	665	427	0.95	(0.74 to 1.23)	63.4
Dose‡§															
No regular use	2138	1359	1.00	(referent)		2053	1274	1.00	(referent)		2465	1516	1.00	(referent)	
Low	320	129	0.66	(0.53 to 0.83)	0.0	439	259	0.96	(0.79 to 1.16)	11.7	113	68	1.15	(0.84 to 1.59)	0.0
High	415	211	0.89	(0.73 to 1.08)	0.0	490	233	0.76	(0.64 to 0.91)	0.0	500	293	0.90	(0.68 to 1.19)	60.4
Duration‡															
No regular use	6625	3667	1.00	(referent)		6451	3568	1.00	(referent)		7106	3918	1.00	(referent)	
<60 months	819	401	0.83	(0.68 to 1.01)	42.3	1002	490	0.86	(0.71 to 1.04)	48.6	477	243	0.88	(0.72 to 1.08)	26.5
≥60 months	833	527	0.98	(0.86 to 1.11)	0.0	824	525	1.08	(0.86 to 1.34)	55.6	712	438	1.13	(0.92 to 1.39)	44.4

* NSAID = nonsteroidal anti-inflammatory drug.

† Summary odds ratios were estimated using random-effects meta-analytic model. Results were adjusted for age (<50, 50–54, 55–59, 60–64, 65–69, ≥70 years), race (white, black, other), oral contraceptive use (ever/never), parity (0, 1, ≥2), menopausal status (premenopausal/postmenopausal), body mass index category (<25, 25–29.9, ≥30 kg/m^2) if available, and first-degree family history of breast cancer, male breast cancer, or ovarian cancer. All statistical tests were two-sided.

‡ Analyses included seven studies for frequency (13,23,26,37–40), three studies for dose (37,38,40), and eight studies for duration.(13,23,34,37–39,42,43).

§ Dose categories for aspirin: low: <100 mg, high: ≥100 mg; for nonaspirin NSAIDs and acetaminophen: low: <500 mg, high: ≥500 mg.

‖ I^2 is the percentage of variation across studies due to heterogeneity rather than chance.

Table 3. Summary odds ratios (ORs) and 95% confidence intervals (CIs) for the associations of aspirin and NSAID use with risk of ovarian cancer in the Ovarian Cancer Association Consortium (1992–2009)*

	Aspirin					Nonaspirin NSAID				
Exposure categorization	**Control**	**Case**	**OR†**	**(95% CI)**	**I^2§**	**Control**	**Case**	**OR†**	**(95% CI)**	**I^2§**
Frequency and dose‡										
No regular use	2138	1359	1.00	(referent)		2053	1274	1.00	(referent)	
<30 days per month, low dose	19	11	1.12	(0.52 to 2.43)	0.0	175	115	1.08	(0.74 to 1.59)	52.1
Daily, low Dose	298	118	0.64	(0.50 to 0.81)	0.0	263	143	0.88	(0.70 to 1.11)	0.0
<30 days per month, high dose	93	66	1.25	(0.88 to 1.76)	0.0	136	82	0.77	(0.57 to 1.04)	0.0
Daily, high Dose	322	144	0.78	(0.62 to 0.97)	0.0	353	148	0.75	(0.60 to 0.94)	3.8

* NSAID = nonsteroidal anti-inflammatory drug.

† Summary odds ratios were estimated using random-effects meta-analytic model. Results were adjusted for age (<50, 50–54, 55–59, 60v64, 65–69, ≥70 years), race (white, black, other), oral contraceptive use (ever/never), parity (0, 1, ≥2), menopausal status (premenopausal/postmenopausal), body mass index category (<25, 25–29.9, ≥30 kg/m^2) if available, and first-degree family history of breast cancer, male breast cancer, or ovarian cancer. All statistical tests were two-sided.

‡ Analyses included three studies for frequency and dose analyses (37,38,40). Dose categories for aspirin: low: <100 mg, high: ≥100 mg; for nonaspirin NSAIDs and acetaminophen: low: <500 mg, high: ≥500 mg.

§ I^2 is the percentage of variation across studies due to heterogeneity rather than chance.

However, daily and/or low-dose aspirin use was not specifically evaluated in the meta-analysis. In contrast, the use of individual-level data in this study facilitated the evaluation of usage patterns beyond what was available in the meta-analysis of published studies.

The pharmacological effects of NSAIDs that lead to reduced risks of cancer or improved cancer prognosis are not well understood and may differ by cancer site. Aspirin is a strong, irreversible inhibitor of COX-1. Nonaspirin NSAIDs are nonselective and reversible inhibitors of both COX-1 and COX-2, whereas acetaminophen is a more effective inhibitor of COX-2 (52,53). The different effects observed in our study for aspirin/nonaspirin NSAIDs and acetaminophen may suggest that COX-1 inhibition is important for ovarian cancer risk reduction, a notion that is further supported by frequent overexpression of COX-1 in ovarian cancer tissue, but more biological and pharmacological research is needed to understand the underlying mechanisms (54).

Both epidemiologic studies and randomized trials have reported inverse associations between aspirin use and colorectal cancer, with a relative risk of approximately 0.5 for regular users (55). There is some evidence that regular and prolonged aspirin use is also associated with reduced risk of cancers of the esophagus (16), bladder (56), liver (57), lung (16), endometrium (58), and female breast (16). A recent pooled analysis of individual patient data from 51 randomized trials of aspirin use for cardiovascular disease prevention reported a 12% reduction in cancer incidence with 3 or more years of daily aspirin use (14). In women, the reduction in incidence was greatest for cancers of the female reproductive organs; however, ovarian cancer incidence was very low (14).

In the Women's Health Study, use of low-dose aspirin every other day was not associated with reduced incidence of colorectal cancer or cancer overall, suggesting that a daily use regimen is important for cancer protection (59). This notion is supported by our findings: the reduction of ovarian cancer risk was much stronger when daily use was considered, and the strongest reduction was observed among daily users of low-dose aspirin. This finding is likely explained by the regular use pattern of low-dose aspirin because low-dose aspirin regimens for cardiovascular protection are characterized by daily use over a long period of time.

Quantifying desired and adverse effects of aspirin will be important when evaluating future public health decisions about aspirin use for prevention of cardiovascular disease and cancer. Complications associated with aspirin use, including peptic ulcer, upper gastrointestinal bleeding, and hemorrhagic stroke, pose serious threats; current risk–benefit analyses favor aspirin use among high-risk groups but not for large-scale, population-based chemoprevention. Our study provides estimates on the effect of aspirin on ovarian cancer risk that should be considered in risk–benefit analyses for preventive aspirin use. However, detailed questions about frequency, dose, and duration will need to be evaluated in future studies including pooled data from cohort studies.

This pooled analysis of data from 12 studies offered several notable strengths. With more than 7500 case patients, we had greater power to detect associations than in any previous single study. Further, we were able to consistently adjust for potential confounders across studies and to evaluate NSAID exposure compared with a common reference group, reducing exposure misclassification (23). Observing consistent associations across studies and countries provided additional robustness to our findings, specifically for aspirin use, where the interstudy heterogeneity was the smallest. The use of individual-level data and the ability to consider and control for a wide range of potential confounders were additional strengths of this pooled analysis.

Potential limitations include possible differential recall of medication use between case patients and control subjects. However, the decreased risk observed for aspirin or nonaspirin NSAIDs and the lack of association with acetaminophen argues against substantial differential recall. Further, the study-specific prevalence of regular aspirin use in the US studies (11%–16%) included in the current analysis is consistent with estimates reported in US cohorts (60–62); differential recall (ie, greater reporting of medication use among case patients) would have biased our results toward the null. There was evidence of heterogeneity between study-specific estimates, but this was mostly restricted to analyses pertaining to nonaspirin NSAIDs and acetaminophen use. Nonaspirin NSAIDs include a variety of drugs and formulations with regional differences that may have contributed to heterogeneity. Another limitation of this pooled analysis was the variability in the definition of regular use across study

Table 4. Summary odds ratios (ORs) and 95% confidence intervals (CIs) for the associations of aspirin, nonaspirin NSAID, and acetaminophen/paracetamol use with risk of ovarian cancer subtype in the Ovarian Cancer Association Consortium (1992–2009)*

Subtype	Aspirin					Nonaspirin NSAID					Acetaminophen				
	Controls	Cases	OR†	(95% CI)	I^2‡	Controls	Cases	OR†	(95% CI)	I^2‡	Controls	Cases	OR†	(95% CI)	I^2‡
Serous															
No regular use	9501	3622	1.00	(referent)		8940	3467	1.00	(referent)		9326	3478	1.00	(referent)	
Use	2123	769	0.89	(0.80 to 0.99)	4.3	2754	1002	0.83	(0.68 to 1.02)	75.4	1878	777	1.03	(0.91 to 1.18)	33.3
Endometrioid															
No regular use	9460	951	1.00	(referent)		8903	858	1.00	(referent)		9264	920	1.00	(referent)	
Use	2115	183	0.90	(0.74 to 1.09)	5.5	2742	290	0.93	(0.75 to 1.15)	38.8	2277	192	0.83	(0.66 to 1.05)	29.1
Clear cell															
No regular use	8800	507	1.00	(referent)		8215	456	1.00	(referent)		9070	510	1.00	(referent)	
Use	1906	110	1.09	(0.84 to 1.41)	9.1	2561	169	0.97	(0.73 to 1.27)	35.0	3222	166	1.22	(0.91 to 1.64)	32.7
Mucinous															
No regular use	8897	308	1.00	(referent)		8340	270	1.00	(referent)		8927	314	1.00	(referent)	
Use	2312	62	0.89	(0.58 to 1.38)	38.1	2625	96	0.99	(0.73 to 1.35)	21.0	1987	66	0.90	(0.66 to 1.23)	0.0

* NSAID = nonsteroidal anti-inflammatory drug.

† Summary odds ratios were estimated using random-effects meta-analytic model. Results were adjusted for age (<50, 50–54, 55–59, 60–64, 65–69, ≥70 years), race (white, black, other), oral contraceptive use (ever/never), parity (0, 1, ≥2), menopausal status (premenopausal/postmenopausal), body mass index category (<25, 25–29.9, ≥30 kg/m^2) if available, and first-degree family history of breast cancer, male breast cancer, or ovarian cancer. All statistical tests were two-sided.

‡ I^2 is the percentage of variation across studies due to heterogeneity rather than chance.

populations. We addressed the misclassification of exposure definitions across the studies by using a standard definition for regular use as described in the Methods; in the two studies with the least restrictive definition of regular use (26,37), participants were reclassified accordingly. We conducted a sensitivity analysis restricting the pooled analysis to those studies with regular use for at least 6 or more months in duration and found similar results. We were not able to reclassify participants from two studies with the most restrictive definition of regular use (23,45). In a sensitivity analysis excluding these two studies from the pooled analysis, the results were essentially unchanged. The details of NSAID use patterns ascertained in each study population differed, and data on frequency, dose, and duration of use were not provided in all studies; thus some subgroup analyses are based on small numbers. Although the point estimates for duration of use suggest a counterintuitive trend of shorter duration of use associated with lower risk of ovarian cancer, the differences were not statistically significant. It will be important to follow up the findings in large pooled prospective studies to better understand the effects of duration and timing of aspirin use and ovarian cancer risk. Further, we were not able to evaluate indication of use.

In summary, this pooled analysis supports the hypothesis that regular aspirin use reduces ovarian cancer risk. Specifically, we report a statistically significant decreased risk of ovarian cancer with daily use of aspirin. Further biological and pharmacological research is necessary to understand the mechanisms of ovarian cancer risk reduction by aspirin use.

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Funding

The Ovarian Cancer Association Consortium was supported by a grant from the Ovarian Cancer Research Fund thanks to donations by the family and friends of Kathryn Sladek Smith. This work was supported in part by the Intramural Research Program of the National Institutes of Health (NIH). Elizabeth Poole and Megan Murphy are both supported in part by training grant T32 CA 09001. Ellen Goode is supported by R01 CA122443 and P50-CA136393. The Australian Ovarian Cancer Study and Australian Cancer Study were funded by the US Army Medical Research and Material Command (DAMD17-01-1-0729), National Health and Medical Research Council of Australia (199600, 400413), Cancer Councils of New South Wales, Victoria, Queensland, South Australia and Tasmania, Cancer Foundation of Western Australia. The Connecticut Ovarian Cancer Study was funded by R01 CA074850 and R01 CA080742. The Diseases of the Ovary and their Evaluation Study was funded by R01 CA112523 and R01 CA87538. The Hawaii Ovarian Cancer Case–Control Study was funded by R01 CA58598, N01 CN55424 and N01 PC67001. The Hormones and Ovarian Cancer Prediction Study was funded by R01 CA95023 and Department of Defense (DOD) grant DAMD17-02-1-0669. The Malignant Ovarian Cancer Study was funded by R01 CA61107, research grant 94 222 52 from the Danish Cancer Society, Copenhagen, Denmark, and the Mermaid I project. The North Carolina Ovarian Cancer Study was funded by R01 CA76016 and DOD grant DAMD17-02-1-0666. The New England Case–Control Study of Ovarian Cancer was funded by R01 CA54419, P50 CA105009, and DOD grant W81XWH-10-1-02802. The New Jersey Ovarian Cancer Study was funded by the National Cancer Institute (K07 CA095666, R01 CA83918, and K22CA138563) and the Cancer Institute of New Jersey. The University of California, Irvine Ovarian Cancer Study was funded by R01 CA58860, R01 CA92044, PSA 042205, and the Lon V Smith Foundation grant LVS-39420. The United Kingdom Ovarian Cancer Population Study was funded by Cancer Research UK, the Eve Appeal and the OAK Foundation. The University of Southern California, Study of Lifestyle and Women's Health was funded by R01 CA17054, R01 CA14089, R01 CA61132, N01-PC-67010, P01 CA17054, California Cancer Research Program (00-01389V-20170, R03 CA113148, R03 CA115195, N01 CN25403), and California Cancer Research Program (2II0200) (USC). The funding agencies did not have any role in conducting the study or preparing the manuscript for publication.

Notes

The Australian Ovarian Cancer Study Management Group (D. Bowtell, G. Chenevix-Trench, A. deFazio, D. Gertig, A. Green, P. Webb) and Australian Cancer Study investigators (A. Green, P. Parsons, N. Hayward, P. Webb, D. Whiteman) thank all the clinical and scientific collaborators (see http://www.aocstudy.org/) and the women for their contribution. The cooperation of the 32 Connecticut hospitals, including Stamford Hospital, in allowing patient access, is gratefully acknowledged. This study was approved by the State of Connecticut Department of Public Health Human Investigation Committee. Certain data used in this study were obtained from the Connecticut Tumor Registry in the Connecticut Department of Public Health. The authors assume full responsibility for analyses and interpretation of these data. The MALOVA group is grateful to Nick Martinussen for data management assistance. The NJO group thanks Drs Lorna Rodriguez and Lisa Paddock, the staff of the New Jersey State Cancer Registry, and Thanusha Puvananayagam for their contribution to the study. Some of this work was undertaken at University College London Hospital/University College London, which received a proportion of funding from the Department of Health's National Institutes for Health Research Biomedical Research Centre funding scheme. We particularly thank I. Jacobs, M. Widschwendter, E. Wozniak, A. Ryan, J. Ford and N. Balogun for their contribution to the study.

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Exhibit 107

JNCI J Natl Cancer Inst (2019) 111(2): djy100

doi: 10.1093/jnci/djy100
Article

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Analgesic Use and Ovarian Cancer Risk: An Analysis in the Ovarian Cancer Cohort Consortium

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Abstract

Background: Aspirin use is associated with reduced risk of several cancers. A pooled analysis of 12 case control studies showed a 10% decrease in ovarian cancer risk with regular aspirin use, which was stronger for daily and low dose users. To prospectively investigate associations of analgesic use with ovarian cancer, we analyzed data from 13 studies in the Ovarian Cancer Cohort Consortium (OC3).

Methods: The current study included 758 829 women who at study enrollment self reported analgesic use, among whom 3514 developed ovarian cancer. Using Cox regression, we assessed associations between frequent medication use and risk of ovarian cancer. Dose and duration were also evaluated. All statistical tests were two sided.

Results: Women who used aspirin almost daily (≥6 days/wk) vs infrequent/nonuse experienced a 10% reduction in ovarian cancer risk (rate ratio [RR] 0.90, 95% confidence interval [CI] 0.82 to 1.00, P .05). Frequent use (≥4 days/wk) of aspirin (RR 0.95, 95% CI 0.88 to 1.03), nonaspirin nonsteroidal anti inflammatory drugs (NSAIDs; RR 1.00, 95% CI 0.90 to 1.11), or acetaminophen (RR 1.05, 95% CI 0.88 to 1.24) was not associated with risk. Daily acetaminophen use (RR 1.28, 95% CI 1.00 to 1.65, P .05) was asso ciated with elevated ovarian cancer risk. Risk estimates for frequent, long term (10+ years) use of aspirin (RR 1.15, 95% CI 0.98 to 1.34) or nonaspirin NSAIDs (RR 1.19, 95% CI 0.84 to 1.68) were modestly elevated, although not statistically significantly so.

Conclusions: This large, prospective analysis suggests that women who use aspirin daily have a slightly lower risk of developing ovarian cancer (~10% lower than infrequent/nonuse) similar to the risk reduction observed in case control anal yses. The observed potential elevated risks for 10+ years of frequent aspirin and NSAID use require further study but could be due to confounding by medical indications for use or variation in drug dosing.

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Ovarian cancer is the most fatal gynecologic cancer, largely due to delayed symptom presentation and lack of early detection strategies. Chemoprevention has not been widely studied but may present approaches to reduce ovarian cancer burden. Chronic inflammation likely plays a key role in ovarian carcino genesis (1). Factors associated with epithelial disruption

Received: January 15, 2018; **Revised:** March 14, 2018; **Accepted:** April 30, 2018

through ovulation (2,3), inflammation related exposures such as endometriosis and pelvic inflammatory disease (4,5), and cir culating biomarkers of inflammation (6,7) have been associated with ovarian cancer risk.

Inhibition of cyclooxygenase (COX) enzymes in prosta glandin synthesis is a primary mechanism responsible for the anti inflammatory and antineoplastic effects of nonste roidal anti inflammatory drugs (NSAIDs) (8,9), and may play a role in ovarian carcinogenesis. Additionally, NSAIDs may suppress ovulation and affect cell proliferation, angiogene sis, and apoptosis of the epithelium (10). Acetaminophen, another common analgesic and antipyretic, has weak anti inflammatory activity and antigonadotropic effects (11). It also may inhibit ovarian carcinogenesis through the deple tion of glutathione leading to necrosis (12). Aspirin, nonas pirin NSAIDs, and acetaminophen are widely used, so any increased or decreased cancer risk may have important pub lic health implications.

Cardiovascular disease prevention trials have shown that daily aspirin use is associated with reduced risk and mortality of several malignancies (eg, colorectal cancer) (13). However, the limited number of women in these trials is insufficient to evalu ate ovarian cancer end points (14).

A recent pooled analysis of 12 case control studies in the Ovarian Cancer Association Consortium (OCAC) reported a re duced risk of ovarian cancer with aspirin use, particularly for daily aspirin users (15). High dose nonaspirin NSAID use, but not acetaminophen, was also associated with lower risk (15). The few prospective observational studies between aspirin or other NSAID use and ovarian cancer risk have had inconsistent results (16 20). Prospective studies avoid potential biases that may occur in case control studies, including differences be tween nonresponders and responders among cases or controls or differences in recollection or reporting of medication use af ter being diagnosed with ovarian cancer. However, the de creased risk observed for aspirin or nonaspirin NSAIDs and the lack of association with acetaminophen in case control studies argues against substantial differential recall (15). Further, the exposure window being evaluated in case control studies is of ten shortly before cancer diagnosis, during which use may be influenced by preclinical disease. Prospective assessment of an algesic use many years before ovarian cancer diagnosis is nec essary to confirm the association with an eye toward improving prevention recommendations. Thus, we evaluated the associa tion between frequent aspirin, nonaspirin NSAID, and acet aminophen use with ovarian cancer risk using prospective individual level data from the Ovarian Cancer Cohort Consortium (OC3).

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Methods

Study Population

The study population included women participating in 16 pro spective cohort studies from North America and Europe (Supplementary Table 1, available online) (16,17,19,21 35). Eligible studies were a cohort study or clinical trial with pro spective follow up including women, determination of ovarian cancer end points through questionnaire/medical record follow up or confirmation by cancer registries, and follow up for death. This analysis was limited to 13 studies that collected information on frequent aspirin, nonaspirin NSAID, or acet aminophen (paracetamol) use over at least a six month period (n = 758 829). All studies obtained institutional approval at their respective institutions; participants provided either written in formed consent or implicit consent through return of the study questionnaire. The OC3 Data Coordinating Center and analytic approaches were approved by the institutional review board of the Brigham and Women's Hospital.

Exposure Definitions

Medication use was self reported at enrollment (Supplementary Table 1, available online) (16,17,19,21,22,24 27,29 34). Given the rationale for assessment of frequent use based on biologic mechanisms and published research (13 15), we focused on fre quent medication use (at least 4 5 days/wk) when possible. Frequency was available in 10 of 13 studies (16,17,21,24,25,29 32), whereas three studies included frequency in their definition of regular medication use (19,22,26). Frequent use was defined as use at least four to five times per week for at least six months' duration; less frequent use or nonregular use/no use were combined to form the reference group. We also evaluated very frequent (daily/almost daily) use for at least 6 months' dura tion as one of the following: six to seven days per week, seven days per week, or 28 or more days per month (11 studies) (16,17,21,22,24,25,29 32). Frequency variables were further di vided by duration of use (all medications: $\geq$0.5 5, >5 10, >10 years, 9 studies) (16,19,24 26,30 32) and aspirin dose (<100 [or "baby aspirin"] and $\geq$100 mg, four studies) (16,19,23,31).

Potential confounding variables were harmonized from the studies as part of a core data set. A priori adjustment factors in cluded baseline age (continuous), body mass index (<20, 20 24.9, 25 29.9, 30 34.9, $\geq$35 kg/m^2), number of births (0, 1, 2, 3, $\geq$4 full term births), duration of oral contraceptive (OC) use (never, $\leq$1, >1 5, >5 10, >10 years), and menopause/duration of meno pausal hormone therapy (premenopausal, postmenopausal: never, $\leq$5, >5 10, >10 years).

Outcome Definitions

We included epithelial ovarian or peritoneal tumors identified either through cancer registries or medical record review (ICD9 codes 183 and 158; ICD10 codes C56). We first evaluated associ ations of medications with all tumors combined (ovarian and peritoneal, n = 3514). Second, we evaluated associations for in vasive epithelial ovarian cancers (n = 3147), and, third, we evaluated associations for the four most common tumor his totypes: serous (n = 1475, including tumors coded as poorly differentiated), endometrioid (n = 233), mucinous (n = 125), and clear cell (n = 111). The remaining 1203 cases had another histology (eg, mixed) or were missing histology information (n = 817) and were censored at diagnosis date in histology specific analyses.

Statistical Methods

Women were excluded from primary analyses if they had a his tory of cancer (other than nonmelanoma skin cancer) at base line, bilateral oophorectomy before study entry, or were missing age. We calculated hazard ratios (HRs) and 95% confidence intervals (CIs) using Cox proportional hazards regression to evaluate the association between the analgesic medications and risk of ovarian cancer. Women entered the analysis at age at study entry and contributed person time until the age at first diagnosis of ovarian cancer (event), death (censored), or end of

follow up (censored), whichever came first. In primary analyses, we pooled data from all cohorts, stratifying on cohort to account for potential differences in baseline hazards. Secondarily, we used meta analysis of cohort specific estimates to assess between study heterogeneity. Associations between analgesic medication use and ovarian cancer histotype were calculated using competing risks Cox regression (36). Statistical heterogeneity of associations across histotypes was assessed via likelihood ratio test comparing a model that assumed different associations for the exposure of interest by histotype (full model) with a model with a single estimate across histotypes (reduced model) (37).

Effect modification by factors that influence inflammation (eg, smoking, body mass index [BMI], history of chronic disease) and established ovarian cancer risk factors (eg, age, parity, OC use, endometriosis) was evaluated using multiplicative interaction terms, with statistical significance assessed by a likelihood ratio test.

In sensitivity analyses, we considered a common reference group, coding "nonfrequent users" as women who reported no or infrequent use of aspirin, nonaspirin NSAIDs, and acetaminophen to account for analgesic usage patterns. We also excluded women who reported a history of chronic disease at baseline to assess potential indication for medication use and explored the potential for reverse causation by evaluating associations of frequent analgesic use with ovarian cancer cases that occurred less than five years, five to less than 10 years, and 10 or more years after baseline. Another sensitivity analysis considered death a competing risk (rather than censoring). Exposure curves from survivor function plots were parallel, suggesting no deviation from proportional hazards. All statistical tests were two sided, and *P* values of less than .05 were considered statistically significant; analyses were performed using SAS 9.1.

Results

Study Characteristics

The proportion of women reporting frequent analgesic use increased with age; for example, among women reporting frequent aspirin use, 17.7% were younger than age 50 years, whereas 52.2% were 60 years of age or older (Table 1). Compared with women who did not use aspirin or who used it infrequently, women who frequently used aspirin were more likely to be older, be postmenopausal, have a history of a chronic disease, have higher BMI, and were less likely to have previously used OCs. Average follow up after exposure assessment was 10.8 years (maximum = 18.9 years); individual cohort follow up is reported in Supplementary Table 1 (available online).

Aspirin

Women who used aspirin at least four to five times per week (n = 851 exposed cases [events]) developed ovarian cancer at about the same rate as women who did not use aspirin or used it only infrequently (HR = 0.95, 95% CI = 0.88 to 1.03) (Table 2). However, compared with infrequent/nonusers, women reporting daily or almost daily use (at least 6 days/wk or more, n = 449 cases) had a 10% reduction in ovarian cancer risk (HR = 0.90, 95% CI = 0.82 to 1.00, *P* = .05). This association was statistically significant for women reporting daily or almost daily use for 0.5 to less than five years' duration (HR = 0.79, 95% CI = 0.63 to 0.99, *P* = .04, n = 87 cases) and was suggestively associated for daily users of five to 10 years' duration (HR = 0.88, 95% CI = 0.65 to 1.18, n = 50 cases). Conversely, women who frequently used (vs infrequent/nonuse) aspirin for long durations (≥10 years at baseline) had a non statistically significantly elevated risk of ovarian cancer (HR = 1.15, 95% CI = 0.98 to 1.34, *P* =.09, n = 212 cases). No associations were observed when analyzing aspirin dose or other patterns of duration. In analyses by histotype (Table 3), results for serous ovarian cancers were similar to those seen for all ovarian cancer: compared with infrequent/nonuse, daily aspirin use was associated with a 15% decrease for serous tumors (95% CI = 0.71 to 1.00, n = 159 cases), whereas 10 or more years of frequent aspirin use was related to a suggestively elevated risk (HR = 1.27, 95% CI = 0.99 to 1.62, n = 74 cases). A similar pattern was observed for clear cell tumors; however, risk estimates were imprecise due to limited numbers. No associations were observed for endometrioid or mucinous tumors.

Nonaspirin NSAIDs

Women who frequently used nonaspirin NSAIDs had a similar rate of ovarian cancer as infrequent/nonusers (HR = 1.00, 95% CI = 0.90 to 1.11, n = 426 cases) (Table 2). Longer duration or daily frequency of nonaspirin NSAIDs was not related to ovarian cancer risk, although the risk estimate for ovarian cancer for frequent, frequent, long duration (>10 years) of use of nonaspirin NSAIDs was suggestively elevated (HR = 1.19, 95% CI = 0.84 to 1.68, n = 36 cases). In analyses by histotype, women who frequently used (vs infrequent/nonuse) nonaspirin NSAIDs for long durations had an increased risk of serous tumors than women who used them infrequently or not at all (HR = 2.06, 95% CI = 1.14 to 3.74, n = 10 cases) (Table 3).

Acetaminophen

Frequent use compared with infrequent/nonuse of acetaminophen was not associated with ovarian cancer risk (HR = 1.05, 95% CI = 0.88 to 1.24, n = 152 exposed cases) (Table 2). However, there was a suggestive elevated risk with daily acetaminophen use (HR = 1.28, 95% CI = 1.00 to 1.65, *P* = .05, n = 71 cases) that was stronger for serous tumors (HR = 1.70, 95% CI = 1.14 to 2.55, n = 26 cases) (Table 3).

Additional Analyses

There was little heterogeneity across studies (data not shown). Risk estimates were generally similar across age strata (Supplementary Table 2, available online). Compared with infrequent/nonusers, daily aspirin use was related to reduced ovarian cancer risk among women younger than age 50 years (HR = 0.89, 95% CI = 0.43 to 1.84), age 50 to 59 years (HR = 0.92, 95% CI = 0.73 to 1.17), and age 60 to 69 years (HR = 0.88, 95% CI = 0.75 to 1.04) at baseline but was null for women age 70 years or older (HR = 1.05, 95% CI = 0.82 to 1.36, $P_{interaction}$ = .73). Daily acetaminophen use was only associated with increased ovarian cancer risk among women age 70 years or older (HR = 1.78, 95% CI = 1.17 to 2.72, $P_{interaction}$ < .001). Results were similar across strata of other ovarian cancer risk factors (data not shown).

Results were similar in analyses restricted to invasive ovarian cancers, utilizing a common reference group, and accounting for death as a competing risk (data not shown). In analyses excluding women with a history of chronic disease, elevated risk estimates with frequent long duration use of aspirin or

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Table 1. Distribution of frequent analgesic use by baseline demographic and health characteristics in the Ovarian Cancer Cohort Consortium (n = 758 829)

	Aspirin		Nonaspirin NSAID		Acetaminophen	
Characteristics	Infrequent/nonuse No. (%)	Frequent use* No. (%)	Infrequent/nonuse No. (%)	Frequent use* No. (%)	Infrequent/nonuse No. (%)	Frequent use* No. (%)
Age, mean (SD), y	54.7 (11.4)	59.4 (10.1)	59.1 (9.5)	59.6 (8.5)	57.7 (10.6)	60.9 (10.0)
Age, y						
<50	171 049 (31.0)	28 462 (17.7)	68 208 (15.8)	10 496 (12.9)	69 762 (22.9)	3973 (14.4)
50 59	182 326 (33.0)	48 432 (30.1)	144 873 (33.6)	29 425 (36.1)	101 553 (33.4)	8351 (30.3)
60+	198 689 (36.0)	84 044 (52.2)	218 295 (50.6)	41 609 (51.0)	132 697 (43.6)	15 244 (55.3)
BMI, kg/m^2						
<20	38 712 (7.0)	9460 (5.9)	28 981 (6.7)	3239 (4.0)	20 937 (6.9)	1513 (5.5)
20 24.9	246 476 (44.6)	63 791 (39.6)	183 064 (42.4)	25 614 (31.4)	127 806 (42)	9216 (33.4)
25 29.9	157 968 (28.6)	49 716 (30.9)	130 232 (30.2)	25 969 (31.9)	89 960 (29.6)	8560 (31.1)
30 34.9	61 441 (11.1)	21 816 (13.6)	51 919 (12.0)	14 072 (17.3)	36 797 (12.1)	4342 (15.8)
35+	33 201 (6.0)	12 620 (7.8)	26 604 (6.2)	10 813 (13.3)	21 015 (6.9)	3068 (11.1)
Missing	14 266 (2.6)	3535 (2.2)	10 576 (2.5)	1823 (2.2)	7497 (2.5)	869 (3.2)
Age at menarche, y						
≤11	129 521 (23.5)	39 029 (24.3)	104 278 (24.2)	22 428 (27.5)	58 358 (19.2)	5549 (20.1)
12	132 550 (24.0)	43 314 (26.9)	107 177 (24.8)	22 151 (27.2)	82 000 (27.0)	8085 (29.3)
13	155 896 (28.2)	42 510 (26.4)	122 489 (28.4)	19 967 (24.5)	87 684 (28.8)	6628 (24.0)
14	71 928 (13.0)	21 378 (13.3)	55 615 (12.9)	10 314 (12.7)	44 990 (14.8)	4640 (16.8)
≥15	48 479 (8.8)	13 304 (8.3)	38 367 (8.9)	6361 (7.8)	27 904 (9.2)	2428 (8.8)
Missing	13 690 (2.5)	1403 (0.9)	3450 (0.8)	309 (0.4)	3076 (1.0)	238 (0.9)
Duration, oral contraceptive use, y						
Never	210 399 (38.1)	79 036 (49.1)	193 635 (44.9)	32 992 (40.5)	112 760 (37.1)	11 756 (42.6)
>0 1	43 208 (7.8)	14 589 (9.1)	32 672 (7.6)	7606 (9.3)	27 743 (9.1)	2557 (9.3)
>1 5	97 165 (17.6)	24 065 (15.0)	67 121 (15.6)	13 458 (16.5)	47 757 (15.7)	3612 (13.1)
>5 10	78 116 (14.1)	16 254 (10.1)	48 201 (11.2)	9520 (11.7)	36 471 (12.0)	2323 (8.4)
>10	104 143 (18.9)	24 316 (15.1)	76 349 (17.7)	16 530 (20.3)	65 839 (21.7)	6257 (22.7)
Missing	19 033 (3.4)	2678 (1.7)	13 398 (3.1)	1424 (1.7)	13 442 (4.4)	1063 (3.9)
No. of pregnancies						
0	85 920 (15.6)	16 579 (10.3)	56 916 (13.2)	9977 (12.2)	42 630 (14.0)	2899 (10.5)
1	60 572 (11.0)	14 426 (9.0)	45 993 (10.7)	8030 (9.8)	35 178 (11.6)	2988 (10.8)
2	177 064 (32.1)	44 857 (27.9)	128 389 (29.8)	23 169 (28.4)	97 780 (32.2)	7997 (29.0)
3	131 053 (23.7)	42 162 (26.2)	110 188 (25.5)	21 291 (26.1)	67 767 (22.3)	6372 (23.1)
4+	93 130 (16.9)	41 287 (25.7)	85 208 (19.8)	17 992 (22.1)	55 969 (18.4)	6706 (24.3)
Missing	4325 (0.8)	1627 (1.0)	4682 (1.1)	1071 (1.3)	4688 (1.5)	606 (2.2)
Menopausal status						
Premenopausal	188 738 (34.2)	31 168 (19.4)	83 184 (19.3)	12 792 (15.7)	82 248 (27.1)	3986 (14.5)
Postmenopausal	348 494 (63.1)	125 619 (78.1)	342 938 (79.5)	67 335 (82.6)	216 731 (71.3)	22 957 (83.3)
Missing	14 832 (2.7)	4151 (2.6)	5254 (1.2)	5254 (1.7)	5033 (1.7)	625 (2.3)
Age at menopause among postmenopausal women, y						
≤45	45 905 (12.6)	15 523 (12.0)	45 476 (13.1)	8341 (12.1)	33 314 (15.0)	3162 (13.4)
46 50	89 057 (24.5)	32 661 (25.2)	86 398 (24.8)	15 875 (23.1)	60 363 (27.2)	6024 (25.5)
51 55	123 290 (33.9)	43 577 (33.6)	125 242 (36.0)	22 357 (32.5)	77 772 (35.1)	7313 (31.0)
>55	24 452 (6.7)	9294 9294 (7.2)	25 889 (7.4)	5503 (8.0)	14 587 (6.6)	1600 (6.8)
Missing	80 622 (22.2)	28 715 (22.1)	65 187 (18.7)	16 662 (24.2)	35 728 (16.1)	5483 (23.3)
Duration, menopausal hormone use, y						
Never	273 (49.6)	73 279 (45.5)	165 228 (38.3)	26 744 (32.8)	112 911 (37.1)	9282 (33.7)
>0 5	78 (14.3)	29 980 (18.6)	73 431 (17.0)	16 284 (20.0)	54 914 (18.1)	6446 (23.4)
>5 10	43 (7.9)	16 040 (10.0)	41 755 (9.7)	9652 (11.8)	30 399 (10.0)	3512 (12.7)
>10	42 (7.7)	20 700 (12.9)	44 658 (10.4)	13 673 (16.8)	28 174 (9.3)	4487 (16.3)
Missing	113 (20.5)	20 939 (13.0)	106 304 (24.6)	15 177 (18.6)	77 614 (25.5)	3841 (13.9)
History of chronic diseases at baseline included:						
Any cardiovascular disease						
No	19 146 (3.5)	11 630 (7.2)	22 121 (5.1)	8655 (10.6)	26 078 (8.6)	4698 (17.0)
Yes	1763 (0.3)	1545 (1.0)	2500 (0.6)	808 (1.0)	2859 (0.9)	449 (1.6)
Missing	531 155 (96.2)	147 763 (91.8)	406 755 (94.3)	72 067 (88.4)	275 075 (90.5)	22 421 (81.3)
Diabetes						
No	440 316 (85.2)	113 913 (82.0)	308 678 (79.5)	55 152 (81.8)	200 184 (72.8)	14 468 (64.5)
Yes	15 142 (2.9)	9472 (6.8)	16 115 (4.2)	4131 (6.1)	9268 (3.4)	1500 (6.7)
Missing	61 381 (11.9)	15 541 (11.2)	63 500 (16.4)	8161 (12.1)	65 623 (23.9)	6453 (28.8)

(continued)

Table 1. (continued)

Characteristics	Aspirin		Nonaspirin NSAID		Acetaminophen	
	Infrequent/nonuse No. (%)	Frequent use* No. (%)	Infrequent/nonuse No. (%)	Frequent use* No. (%)	Infrequent/nonuse No. (%)	Frequent use* No. (%)
Autoimmune disease						
No	86 690 (18.2)	35 539 (25.5)	104 565 (28.7)	20 401 (31.8)	115 614 (49.5)	9414 (49.4)
Yes	6192 (1.3)	4179 (3.0)	7292 (2.0)	3159 (4.9)	9630 (4.1)	1855 (9.7)
Missing	383 645 (80.5)	99 626 (71.5)	252 748 (69.3)	40 667 (63.3)	108 156 (46.3)	7787 (40.9)

*Frequent: use at least ~4–5 days/wk for 6 months or longer. BMI body mass index; NSAID nonsteroidal anti-inflammatory drug.

Table 2. Associations between analgesic use and ovarian cancer risk in the Ovarian Cancer Cohort Consortium (n = 758 829)

Analgesic use	No. of events (cases)	Person years	HR* (95% CI)	P†
Aspirin				
Infrequent/nonuse	2404	4 946 886	1.00 (ref)	
Frequent use‡	851	1 408 656	0.95 (0.88 to 1.03)	.23
Frequent use by duration vs infrequent/nonuse				
Infrequent/nonuse	1402	3 150 285	1.00 (ref)	
Frequent/0.5 <5 y	239	504 116	0.92 (0.80 to 1.06)	.24
Frequent/5 <10 y	93	171 582	0.90 (0.72 to 1.12)	.33
Frequent/10+ y	212	305 987	1.15 (0.98 to 1.34)	.09
Categories of frequent use vs infrequent/nonuse				
Infrequent/nonuse	1936	3 245 903	1.00 (ref)	
<Daily use	156	161 238	1.06 (0.90 to 1.26)	.49
Daily use§	449	545 499	0.90 (0.82 to 1.00)	.05
Categories of frequent use by duration vs infrequent/nonuse				
Infrequent/nonuse	1402	3 150 285	1.00 (ref)	
<Daily/0.5 <5 y	152	379 640	1.02 (0.85 to 1.21)	.87
<Daily/5 <10 y	43	108 355	0.92 (0.67 to 1.26)	.60
<Daily/10+ y	113	260 503	1.12 (0.92 to 1.37)	.26
Daily/0.5 <5 y	87	124 476	0.79 (0.63 to 0.99)	.04
Daily/5 10 y	50	63 227	0.88 (0.65 to 1.18)	.39
Daily/10+ y	99	45 484	1.18 (0.93 to 1.50)	.18
Frequent use by dose vs infrequent/nonuse				
Infrequent/nonuse	392	436 742	1.00 (ref)	
Frequent low dose	115	72 719	0.99 (0.79 to 1.23)	.91
Frequent normal dose	144	130 684	0.94 (0.77 to 1.15)	.55
Nonaspirin NSAID				
Infrequent/nonuse	2305	3 798 980	1.00 (ref)	
Frequent use‡	426	614 745	1.00 (0.90 to 1.11)	.96
Frequent use by duration vs infrequent/nonuse				
Infrequent/nonuse	1168	2 051 666	1.00 (ref)	
Frequent/0.5 <5 y	122	237 614	0.94 (0.78 to 1.14)	.54
Frequent/5 <10 y	64	75 230	1.10 (0.85 to 1.42)	.49
Frequent/10+ y	36	29 429	1.19 (0.84 to 1.68)	.33
Categories of frequent use vs infrequent/nonuse				
Infrequent/nonuse	1982	3 049 045	1.00 (ref)	
<Daily use	104	124 937	1.07 (0.88 to 1.31)	.50
Daily use§	237	319 625	0.97 (0.84 to 1.11)	.65
Categories of frequent use vs infrequent/nonuse				
Infrequent/nonuse	1168	2 051 666	1.00 (ref)	
<Daily/0.5 <5 y	83	159 749	1.02 (0.81 to 1.28)	.88
<Daily/5 <10 y	39	43 940	1.31 (0.95 to 1.81)	.10
<Daily/10+ y	15	18 356	1.10 (0.66 to 1.84)	.72
Daily/0.5 <5 y	39	77 865	0.81 (0.58 to 1.14)	.23
Daily/5 <10 y	25	31 290	0.86 (0.57 to 1.30)	.48
Daily/10+ y	21	11 074	1.27 (0.80 to 2.01)	.32

(continued)

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Table 2. (continued)

Analgesic use	No. of events (cases)	Person years	HR* (95% CI)	P†
Acetaminophen				
Infrequent/nonuse	1421	2 583 452	1.00 (ref)	
Frequent use‡	152	213 668	1.05 (0.88 to 1.24)	.61
Frequent use by duration vs infrequent/nonuse				
Infrequent/nonuse	1386	2 425 711	1.00 (ref)	
Frequent/0.5 <5 y	61	95 060	0.99 (0.76 to 1.29)	.93
Frequent/5 <10 y	50	50 683	1.16 (0.87 to 1.54)	.32
Frequent/10+ y	37	51 266	1.01 (0.73 to 1.41)	.96
Categories of frequent use vs infrequent/nonuse				
Infrequent/nonuse	1179	2 120 248	1.00 (ref)	
<Daily use	35	43 645	0.99 (0.70 to 1.39)	.94
Daily use§	71	62 759	1.28 (1.00 to 1.65)	.05
Categories of frequent use by duration vs infrequent/nonuse				
Infrequent/nonuse	1386	2 425 711	1.00 (ref)	
<Daily/0.5 <5 y	33	69 923	0.87 (0.62 to 1.22)	.42
<Daily/5 <10 y	25	35 311	0.98 (0.66 to 1.46)	.93
<Daily/10+ y	22	39 950	0.89 (0.58 to 1.36)	.58
Daily/0.5 <5 y	28	25 137	1.21 (0.81 to 1.81)	.35
Daily/5 <10 y	25	15 372	1.42 (0.94 to 2.13)	.09
Daily/10+ y	15	11 315	1.24 (0.75 to 2.08)	.40

*Hazard ratios and 95% confidence intervals were estimated from Cox proportional hazards models stratified by study cohort and adjusted for baseline age (continuous), body mass index (<20, 20–24.9, 25–29.9, 30–34.9, ≥35 kg/m^2), number of births (none, one, two, three, four or more full–term births), duration of oral contraceptive (OC) use (never, ≤1, >1–5, >5–10, >10 years), and duration of menopausal hormone therapy use (premenopausal, never, ≤5, >5–10, >10 years). CI confidence interval; HR hazard ratio; NSAID nonsteroidal anti-inflammatory drug.

†P value was calculated using a two-sided Wald test.

‡Frequent: use at least ~4–5 days/wk for 6 months or longer.

§Daily: use at least ~6–7 days/wk or ≥28 days per month for 6 months or longer.

nonaspirin NSAIDs were attenuated (aspirin: HR = 1.11, 95% CI = 0.93 to 1.33; nonaspirin NSAIDs: HR = 1.04, 95% CI = 0.68 to 1.60); other associations, including for acetaminophen, remained unchanged. Associations were slightly stronger for frequent long duration use of aspirin or daily acetaminophen use for cases diagnosed within five years of baseline compared with five or more years after baseline (data not shown).

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Discussion

We observed a 10% reduced ovarian cancer risk for daily aspirin use, although only for women who had used aspirin for less than 10 years; use for 10 or more years was associated with a null or slightly elevated risk. Nonaspirin NSAID and acetaminophen use was not clearly related to ovarian cancer risk overall; however, we observed an increased risk for very frequent (daily/almost daily for at least six months) acetaminophen use. Further, like aspirin, long duration, frequent nonaspirin NSAID use was associated, at least suggestively, with elevated risk of ovarian cancer. The modestly reduced risk for daily aspirin use is consistent with previous observations from case control studies (15), although the suggestively elevated risk with long duration of frequent analgesic use requires further evaluation.

Importantly, in this analysis, we were able to evaluate patterns of duration to characterize a dose response association; however, unlike colorectal cancer, in which longer duration of use is associated with further risk reductions (38), the reduced risk of ovarian cancer with frequent aspirin use was only apparent with short to moderate duration (the largest exposure stratum) and appeared null or slightly elevated with longer duration use (≥10 years). This may be because those who frequently used aspirin for many years may be more likely to use standard vs low dose aspirin. That said, availability of data on very long durations of use was limited, as evidenced by the less precise estimates in this group. A better understanding of the relationship between frequency and duration of use leveraging updated exposure data is needed to assess the potential causality of the daily aspirin ovarian cancer relationship, including ascertainment of use during potentially critical time periods given that the increased risk for long duration use was strongest for cases diagnosed early in follow up. Further, consideration of associations for daily aspirin use and its timing/duration with ovarian cancer is needed to fully assess potential for primary prevention, particularly given the relatively low prevalence of ovarian cancer and risk related adverse events (eg, upper gastrointestinal bleeding). Consistent with our results, pooled analyses of clinical trial data demonstrate that daily aspirin use is most relevant for risk reduction of colorectal cancer and cancer risk overall (39), as alternate dosing trials (higher dose or every other day use) did not show clear benefits (40).

The previous pooled case control study and our current study support that daily aspirin use is associated with lower ovarian cancer risk. The weaker association in the prospective studies vs case control studies is similar to results for breast cancer risk (14). Although recall bias may lead to a stronger association in case control studies, we would expect this to attenuate any true reductions in risk with daily aspirin use. Alternately, considering analgesic use collected at study entry may lead to misclassification of exposure status over follow up (which averaged more than a decade long) that could attenuate results. Conversely, we observed a consistently elevated ovarian cancer risk with frequent, long duration use of aspirin and nonaspirin NSAIDs, suggesting potential confounding by medical indications for long term use. We could not directly address this as indication for use was not collected in most studies. To address this in sensitivity analyses, we excluded women who

Table 3. Associations between analgesic use and ovarian carcinoma histologic subtypes, Ovarian Cancer Cohort Consortium

		Serous (n = 1470)		Endometrioid (n = 233)		Mucinous (n = 125)		Clear cell (n = 111)	
Analgesic use	P_{het}*	No. of events	HR† (95% CI)	No. of events	HR† (95% CI)	No. of events	HR† (95% CI)	No. of events	HR† (95% CI)
Aspirin									
Infrequent/nonuse	26	1141	1.00 (ref)	181	1.00 (ref)	93	1.00 (ref)	85	1.00 (ref)
Frequent use‡		307	0.93 (0.81 to 1.05)	45	0.90 (0.64 to 1.27)	29	1.13 (0.73 to 1.75)	25	1.11 (0.71 to 1.74)
Frequent use by duration vs infrequent/nonuse									
Infrequent/nonuse	03	680	1.00 (ref)	132	1.00 (ref)	52	1.00 (ref)	59	1.00 (ref)
Frequent/0.5–<5 y		69	0.85 (0.73 to 0.99)	18	0.93 (0.62 to 1.40)	10	1.03 (0.60 to 1.74)	5	0.75 (0.40 to 1.42)
Frequent/5–<10 y		37	0.89 (0.64 to 1.24)	8	1.28 (0.62 to 2.66)	2	0.67 (0.16 to 2.87)	4	1.46 (0.52 to 4.12)
Frequent/10+ y		74	1.27 (0.99 to 1.62)	8	0.64 (0.31 to 1.31)	10	1.69 (0.83 to 3.42)	10	1.97 (0.98 to 3.97)
Categories of frequent use vs infrequent/nonuse									
Infrequent/nonuse	13	938	1.00 (ref)	139	1.00 (ref)	62	1.00 (ref)	67	1.00 (ref)
<Daily use		57	1.04 (0.86 to 1.25)	3	0.86 (0.55 to 1.34)	1	0.93 (0.53 to 1.63)	4	1.35 (0.75 to 2.41)
Daily use§		159	0.85 (0.71 to 1.00)	20	0.95 (0.59 to 1.54)	14	1.40 (0.77 to 2.56)	9	0.87 (0.44 to 1.73)
Nonaspirin NSAID									
Infrequent/nonuse	06	984	1.00 (ref)	139	1.00 (ref)	67	1.00 (ref)	75	1.00 (ref)
Frequent use‡		157	1.09 (0.92 to 1.30)	18	1.03 (0.61 to 1.73)	8	0.86 (0.41 to 1.77)	6	0.53 (0.23 to 1.22)
Frequent use by duration vs infrequent/nonuse									
Infrequent/nonuse	03	456	1.00 (ref)	71	1.00 (ref)	31	1.00 (ref)	47	1.00 (ref)
Frequent/0.5–<5 y		38	1.01 (0.83 to 1.23)	7	1.09 (0.63 to 1.89)	2	0.84 (0.38 to 1.86)	2	0.54 (0.22 to 1.34)
Frequent/5–<10 y		20	1.39 (0.87 to 2.22)	2	1.04 (0.25 to 4.31)	1	1.51 (0.20 to 11.63)	1	0.71 (0.10 to 4.95)
Frequent/10+ y		10	2.06 (1.14 to 3.74)	0	–	0	–	0	–
Categories of frequent use vs infrequent/nonuse									
Infrequent/nonuse	04	883	1.00 (ref)	115	1.00 (ref)	61	1.00 (ref)	69	1.00 (ref)
<Daily use		38	1.15 (0.87 to 1.53)	7	1.36 (0.61 to 3.00)	3	1.65 (0.65 to 4.20)	1	0.45 (0.11 to 1.83)
Daily use§		102	1.06 (0.86 to 1.31)	9	0.87 (0.45 to 1.67)	3	0.49 (0.15 to 1.58)	4	0.58 (0.21 to 1.59)
Acetaminophen									
Infrequent/nonuse	21	577	1.00 (ref)	103	1.00 (ref)	38	1.00 (ref)	50	1.00 (ref)
Frequent use‡		47	1.29 (0.94 to 1.77)	11	1.77 (0.96 to 3.29)	2	0.70 (0.16 to 2.99)	4	1.49 (0.43 to 5.17)
Frequent use by duration vs infrequent/nonuse									
Infrequent/nonuse	01	557	1.00 (ref)	100	1.00 (ref)	38	1.00 (ref)	46	1.00 (ref)
Frequent/0.5–<5 y		22	1.36 (0.87 to 2.12)	3	0.72 (0.20 to 2.64)	0	–	3	2.42 (0.57 to 10.35)
Frequent/5–<10 y		15	1.44 (0.85 to 2.43)	5	3.66 (1.54 to 8.69)	1	1.68 (0.23 to 12.17)	1	1.48 (0.18 to 11.91)
Frequent/10+ y		8	0.97 (0.48 to 1.96)	3	1.92 (0.58 to 6.32)	1	1.30 (0.16 to 10.48)	0	–
Categories of frequent use vs infrequent/nonuse									
Infrequent/nonuse	09	554	1.00 (ref)	102	1.00 (ref)	35	1.00 (ref)	46	1.00 (ref)
<Daily use		9	0.95 (0.60 to 1.51)	1	1.70 (0.78 to 3.69)	1	1.15 (0.22 to 6.03)	3	1.69 (0.33 to 8.59)
Daily use§		26	1.70 (1.14 to 2.55)	6	1.85 (0.75 to 4.57)	0	–	1	1.15 (0.17 to 8.01)

*The $P_{heterogeneity}$ value was calculated using a two-sided likelihood ratio test (37). CI = confidence interval; HR = hazard ratio; NSAID = nonsteroidal anti-inflammatory drug.

†Hazard ratios and 95% confidence intervals were estimated from competing risk (37). Cox proportional hazards models were stratified on study cohort and adjusted for baseline age (continuous), body mass index (<20, 20–24.9, 25–29.9, 30–34.9, ≥35 kg/m^2), number of births (none, one, two, three, four, or more full-term births), duration of oral contraceptive (OC) use (never, ≤1, >1–5, >5–10, >10 years), and duration of menopausal hormone therapy use (premenopausal, never, ≤5, >5–10, >10 years). Competing risk models were based on fixed covariate effects; variable covariate effect results were practically identical (data not shown).

‡Frequent: use at least ~4–5 days/wk for 6 months or longer.

§Daily: use at least ~6–7 days/wk or ≥28 days per month for 6 months or longer.

reported a history of chronic disease at baseline and observed some attenuation in risk estimates. That said, further assessment of confounding by medical indications for long term use, such as joint pain, osteoarthritis, cardiovascular disease, or other factors, is needed, as well as consideration of potential biologic mechanisms by which long term use may increase risk.

Consistent with our results, acetaminophen use was not associated with ovarian cancer risk in the pooled case control study data (15), based on more than 400 exposed cases (odds ratio for daily vs nonregular use = 0.95, 95% CI = 0.74 to 1.23). Acetaminophen and nonaspirin NSAIDs are commonly used interchangeably; however, acetaminophen has weak anti inflammatory properties and may have gonadotrophic effects (11), supporting the different associations we observed between NSAIDs and acetaminophen in our study and suggesting different anti inflammatory effects or other mechanisms of action (8,9,11). Importantly, the increased risk with daily acetaminophen use observed in this study was based on a limited number of exposed cases and should be interpreted with caution.

The consistent positive relationship for frequent long duration use of aspirin or nonaspirin NSAIDs with serous disease may suggest that long term users likely have other factors that increase inflammation and thus risk of this histotype. Some data suggest that serous tumors may be more strongly related to inflammatory factors. For example, aggressive high grade serous tumors have been more commonly associated with inducible nitric oxide synthase and other inflammatory markers than low grade tumors (41). Further, prediagnostic circulating inflammatory marker, C reactive protein, has been associated with the serous histotype (6,42). Lifetime ovulations also were more strongly associated with tumors expressing p53 (43), a hallmark of serous disease (44).

The prospective design of the pooled studies precludes recall bias. Additional strengths of the study include the large sample size, the ability to identify deaths as well as capture loss to follow up, and the ability to account for many known and suspected risk factors for ovarian cancer. Limitations included the use of self reported exposure data, limited information on low dose aspirin use, and limited data on health conditions or medical indications underlying long term analgesic use. The combination of long term follow up and ascertainment of exposure at baseline (in most studies) mean that individuals could have started or stopped use during follow up, which would contribute to measurement error. Further, information on duration of use at baseline may not adequately represent exposure duration, as such confounding by indication may not fully explain the elevated risks. Residual confounding by age related factors may also be present; however, we did not observe substantial differences in associations across age strata.

The incidence of ovarian cancer is low; thus, our modest findings are unlikely to alter the balance of more common and clinically significant risks and benefits associated with daily aspirin use. However, the associations stratified by age at baseline provide information relevant to current US Preventive Services Task Force recommendations regarding aspirin use for cardiovascular prevention (45), as decreased ovarian cancer risk estimates associated with daily aspirin use were only observed among women younger than age 70 years. The USPSTF does not recommend frequent aspirin use in women age 70 years or older because of increased risk for adverse events. Although the potential increased risk associated with daily acetaminophen and frequent aspirin and nonaspirin NSAID use for more than 10 years' duration requires further study, daily aspirin use may provide a very modestly reduced risk with respect to incident ovarian cancer.

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Funding

This work was supported by Department of Defense Ovarian Cancer Research Program grant W81XWH 12 1 0561. The UKBGS thanks Breast Cancer Now and the Institute of Cancer Research (ICR) for support and funding. The ICR acknowledges National Health Service funding to the National Institute for Health Research Biomedical Research Centre. K05CA154337 from the National Cancer Institute (NCI) and Office of Dietary Supplements (VITAL); R01 CA39742 (Iowa Women's Health Study); research grants from the Swedish Research Council and Swedish Cancer Foundation (SMC, WLHS); UM1 CA164973 (Multiethnic Cohort Study [MEC]); NIH/NCI UM1 CA182876 (SCHS); UM1 CA186107, P01 CA87969, UM1 CA176726, R01 CA67262 (Nurses' Health Study, Nurses' Health Study II); and NIEHS Intramural Research Program (Project Z01 ES044005 to DPS). The Womens Health Initiative (WHI) program is funded by the National Heart, Lung, and Blood Institute, NIH/DHHS, through contracts HHSN268201100046C, HHSN268201100001C, HHSN268201100002C, HHSN268201100003C, HHSN268201100004C, and HHSN271201100004C. NCI Intramural Research Program.

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The funding agency did not have any role in the design of the study; the collection, analysis, or interpretation of the data; the writing of the manuscript; or the decision to submit the manuscript for publication.

Preliminary results from this study were presented at the AACR Rivkin Ovarian Cancer Meeting (September 2016) and at the NCI Cohort Consortium Annual Meeting (November 2016, same abstract).

The authors thank Ruifeng Li for assistance with computer programming and harmonization of covariate data. The UKBGS thanks the study participants, study staff, and the doctors, nurses, and other health care staff and data providers who contributed to the study. We would like to thank the participants and staff of the NHS/NHSII for their valuable contributions and the following state cancer registries for their help: AL, AZ, AR, CA, CO, CT, DE, FL, GA, ID, IL, IN, IA, KY, LA, ME, MD, MA, MI, NE, NH, NJ, NY, NC, ND, OH, OK, OR, PA, RI, SC, TN, TX, VA, WA, WY. The authors assume full responsibility for analyses and interpretation of these data. The NLCS thanks participants and staff who have contributed to the study. The authors thank the WHI investigators for their dedication and the WHI study participants for making the program possible.

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Exhibit 108

FULL PAPER

BJC

British Journal of Cancer (2016) 114, 819–825 | doi: 10.1038/bjc.2016.39

Keywords: ovarian cancer; African American; analgesics; aspirin; non steroidal anti inflammatory drugs; acetaminophen

Analgesic medication use and risk of epithelial ovarian cancer in African American women

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Background: Existing literature examining analgesic medication use and epithelial ovarian cancer (EOC) risk has been inconsistent, with the majority of studies reporting an inverse association. Race specific effects of this relationship have not been adequately addressed.

Methods: Utilising data from the largest population based case control study of EOC in African Americans, the African American Cancer Epidemiology Study, the relationship between analgesic use (aspirin, non aspirin nonsteroidal anti inflammatory drugs (NSAIDs), and acetaminophen) and risk of EOC was estimated by multivariate logistic regression. The association of frequency, duration, and indication of analgesic use on EOC risk was also assessed.

Results: Aspirin use, overall, was associated with a 44% lower EOC risk (OR = 0.56; 95% CI = 0.35 0.92) and a 26% lower EOC risk was observed for non aspirin NSAID use (OR = 0.74; 95% CI = 0.52 1.05). The inverse association was strongest for women taking aspirin to prevent cardiovascular disease and women taking non aspirin NSAIDs for arthritis. Significantly decreased EOC risks were observed for low dose aspirin use, daily aspirin use, aspirin use for <5 years, and occasional non aspirin NSAID use for a duration of $\geqslant 5$ years. No association was observed for acetaminophen use.

Conclusions: Collectively, these findings support previous evidence that any NSAID use is inversely associated with EOC risk.

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Received 9 November 2015; revised 21 January 2016; accepted 28 January 2016; published online 23 February 2016

Inflammation may play a role in ovarian cancer carcinogenesis through the production of toxic oxidants and bioactive substances, increasing the chances of DNA damage and mutagenesis (Ness and Cottreau, 1999). Analgesic drugs, such as aspirin and other nonsteroidal anti inflammatory drugs (NSAIDs), have anti inflammatory properties and have been associated with reduced risks of several malignancies (Schreinemachers and Everson, 1994; García Rodríguez and Huerta Alvarez, 2001; Bosetti *et al*, 2012; Rothwell *et al*, 2012; Neill *et al*, 2013). Another commonly used type of analgesic medication, acetaminophen, has weak anti inflammatory activity, but may reduce cancer risk through antigonadotropic effects (Cramer *et al*, 1998) that may be particularly relevant to ovarian cancer.

The existing literature examining analgesic drug use and ovarian cancer risk is inconsistent, with the majority of studies reporting mild protective associations (Rosenberg *et al*, 2000; Schildkraut *et al*, 2006; Wernli *et al*, 2008; Pinheiro *et al*, 2009) or no association (Moysich *et al*, 2001; Lacey *et al*, 2004; Murphy *et al*, 2012), with few suggesting weak positive relationships (Hannibal *et al*, 2008; Wu *et al*, 2009). A meta analysis of 17 studies concluded that the existing body of evidence does not clearly support the presence of an association between analgesic use and ovarian cancer risk (Ni *et al*, 2013). However, a recent, well powered pooled analysis using data from 12 case control studies participating in the Ovarian Cancer Association Consortium observed a statistically significant decrease in epithelial ovarian cancer (EOC) risk for aspirin use, a decrease in risk for high dose non aspirin NSAID use, and no association for acetaminophen use (Trabert *et al*, 2014).

The majority of published literature examining the relationship between analgesic use and ovarian cancer was conducted in study populations composed predominately of white women, with little representation of African American (AA) women. There are several indications that a differing risk profile may be evident by race; published studies have suggested that AA women have higher inflammatory marker levels (e.g., interleukin 6, C reactive protein) than white women (Albert *et al*, 2004; Khera *et al*, 2005; Paalani *et al*, 2011) and that there are differences in patterns of analgesic use by race (Zhou *et al*, 2014). To our knowledge, only one study (Setiawan *et al*, 2012) has evaluated race specific associations for analgesic use and risk of EOC. Using the Multiethnic Cohort Study, Setiawan *et al* (2012) reported a weak inverse association, although not significant, between analgesic drug use and EOC risk for AA women. Although this is a large prospective study of ~60 000 women, the inferences were limited by the small number of AA ovarian cancer cases ($n=41$). Other studies including AA women had relatively small samples of AA women with inadequate power to assess race specific associations (Schildkraut *et al*, 2006; Wu *et al*, 2009). To overcome these challenges, the present analysis utilised the largest study of EOC in AA women, to date, to examine EOC risk associated with analgesic medication use exclusively among AA women.

MATERIALS AND METHODS

Study population. The African American Cancer Epidemiology Study (AACES) is a population based case control study examining risk factors for EOC exclusively among AA women. The AACES is a collaborative effort between 11 sites, including Alabama, Georgia, Illinois, Louisiana, Michigan, New Jersey, North Carolina, Ohio, South Carolina, Tennessee, and Texas. A detailed description of methods for AACES has been published elsewhere (Schildkraut *et al*, 2014). Briefly, cases were identified through rapid case ascertainment at SEER and state cancer registries, gynaecologic oncology departments, or hospitals. Eligibility criteria for the cases included: self identification of AA race, aged 20 79 years, and newly diagnosed with invasive EOC after December 2010. The AA controls were identified through random digit dialing, and were frequency matched to cases by 5 year age category and state of residence. Women were excluded if previously diagnosed with EOC or if they had a bilateral oophorectomy. The AACES participants completed a telephone interview, including questions on demographic characteristics, reproductive history, oral contraceptive use, hormone therapy, family history of cancers, medication use, and a variety of lifestyle characteristics (e.g., smoking, physical activity). A short form of the questionnaire could also be completed in an effort to increase participation for women who would have otherwise refused. The study protocol was approved by the Institutional Review Board at each site, and informed consent was obtained for all participants.

As of August 2015, AACES has enrolled a total of 593 cases and 750 controls ($N=1343$). Of these, 71 women completed the short questionnaire, 52 cases and 19 controls. As the short form of the questionnaire did not inquire about analgesic medication use, the data set was restricted to include only those women completing the long form of the questionnaire ($N=1272$; 541 cases and 731 controls).

Analgesic drug use. In the questionnaire, participants were asked to recall any medications for pain or inflammation that were taken regularly, defined as at least once a week or at least 5 days out of the month, at any point in their lifetime. Examples of analgesic drugs and indications of use were provided to aid in recollection. Women who responded affirmatively to ever using pain or inflammation medications were then asked the name of the drug (including dosing information, if available), the reason for using the drug, how many days per month taken, age of first and last use, and the duration of use in months or years. This series of questions was repeated if the participant reported using more than one drug in her lifetime, with no participant reporting use of more than 10 analgesic drugs. The names of each drug were reviewed and categorised into the following groups: aspirin, non aspirin NSAIDs, and acetaminophen. Some medications contained a combination of these analgesic types (e.g., aspirin and acetamino phen) and women who reported taking them were categorised as having used both types of analgesics. Any reported medication that did not fit into one of these categories (e.g., muscle relaxants, opioids, anti epileptics) was considered as nonuse. To successfully model a time period of analgesic use that was not influenced by potential symptoms of an undiagnosed EOC, women who reported initiation of analgesic drug use within the year before the reference date (cases: diagnosis of EOC; controls: time of interview) were categorised as nonusers. In addition, a women who reported a duration of use of <6 months or any case who reported initiation of analgesic drug use after her EOC diagnosis was categorised as a nonuser.

Statistical analyses. Multivariate unconditional logistic regression was used to estimate odds ratios (ORs) and 95% confidence intervals (95% CIs) for the relationship between use of analgesic medications and risk of EOC. Each medication type was examined in a separate model with women who never used any analgesic medication serving as the referent group. In addition, the association of any type of NSAID (aspirin and non aspirin NSAIDs, but not acetaminophen) with EOC risk was also examined. Use of aspirin, non aspirin NSAIDs, and acetamino phen was further examined by frequency of use (<30 times per month, daily), duration of use (<5 years, $\geqslant 5$ years), combined frequency and duration of use (<30 times per month for <5 years, daily for <5 years, <30 times per month for $\geqslant 5$ years, daily for $\geqslant 5$ years), and indication of use (arthritis, menstrual cramps, injury/pain, headache, and heart disease). Because of insufficient data on dose for non aspirin NSAIDs and acetaminophen, the

effect of dose on EOC risk was assessed for aspirin only (low dose: <100 mg; high dose: ⩾100 mg).

The following *a priori* confounders were adjusted for in all models: age (age at diagnosis for cases and age at the time of interview for controls); study site (Alabama, Georgia and Tennessee combined (because of small sample sizes and geographic similarities), Illinois and Michigan combined (because of small sample sizes and geographic similarities), Louisiana, New Jersey, North Carolina, Ohio, South Carolina, and Texas); education (high school graduate or less, some post high school training, and college or graduate degree); income (<$25 000, $25 000 $49 999, $50 000 $74 999, and ⩾$75 000); parity (nulliparous, 1, 2, 3, or more live births); family history of a first degree relative with breast or ovarian cancer (yes, no); tubal ligation (yes, no); body mass index (<25 kg m^{-2}: underweight and normal weight; 25 29.9 kg m^{-2}: overweight; ⩾30 kg m^{-2}: obese); oral contraceptive use (ever, never); menopausal status (pre , peri , and post menopause); endometriosis (yes, no); pelvic inflammatory disease (yes, no); mild physical activity (yes, no); and moderate or strenuous physical activity (yes, no). Several comorbid conditions (e.g., heart disease, osteoporosis, and arthritis) were also evaluated as potential confounders, but no appreciable change in the effect estimates was observed and these conditions were not included in the final models. As women commonly reported use of more than one type of analgesic (e.g., of the women reporting acetaminophen use, 40% also reported using non aspirin NSAIDs), use of (frequency of, duration of, indication of) the other analgesic types were simultaneously adjusted for in regression models. Therefore, the estimated associations in the present study reflect the independent effect of that type of analgesic without confounding by use of other analgesic types.

Finally, potential effect modification by two pro inflammatory factors, BMI and smoking, was evaluated to determine whether analgesic medication use may be particularly beneficial for specific subgroups with higher inflammation. All analyses were conducted using SAS, Version 9.3 (SAS, Cary, NC, USA).

RESULTS

A total of 1272 subjects, 541 cases and 731 controls, were included in the analysis. Table 1 describes demographic, reproductive, and lifestyle characteristics of cases and controls. Compared with controls, cases were more likely to be older (because of collapsing of 5 year age categories that were used for frequency matching), to have a high school education or less, to be nulliparous, to have a family history of a first degree relative with breast or ovarian cancer, to have had endometriosis, and to have had pelvic inflammatory disease, and cases were less likely to have had tubal ligation, to use oral contraceptives, and to engage in mild intensity physical activity. The majority of cases were diagnosed with serous EOC (72.8%). Overall, 467 women reported use of any analgesic medication (36.7%). Of those, non aspirin NSAIDs were the most commonly reported analgesic medication (62.7%), followed by aspirin and acetaminophen, 35.1% and 27.4% respectively (percentages do not equal 100% because of women taking more than one type of analgesic drug).

The associations between analgesic medication use and EOC risk are shown in Table 2. In comparison with never users of any analgesic medications, women who used any type of NSAIDs, including aspirin and non aspirin NSAIDs but not acetaminophen, had a 27% lower risk of EOC (OR = 0.73, 95% CI = 0.54 0.98). No association with EOC risk was observed for women who used a combination of all analgesic drug types (OR = 1.03, 95% CI = 0.58 1.84). When evaluating use of each analgesic medication in separate models, a statistically significant 44% lower EOC risk was observed for women who reported use of aspirin (OR = 0.56, 95% CI = 0.35 0.92), and a 26% lower EOC risk (OR = 0.74, 95% CI = 0.52 1.05) was observed for non aspirin NSAID use. Acetaminophen was inversely associated with risk of EOC, although not statistically significant (OR = 0.89, 95% CI = 0.49 1.62).

Table 3 shows the associations for frequency and duration of use for each analgesic drug and risk of EOC. Irrespective of frequency and duration of aspirin use, inverse associations with risk of EOC were observed. Statistically significant lower risks of EOC were observed for daily aspirin use (OR = 0.56, 95% CI = 0.34 0.94) and aspirin use for a duration of <5 years (OR = 0.52, 95% CI = 0.28 0.98). A significant inverse association with risk of EOC was observed for AA women who occasionally used non aspirin NSAIDs (<30 days per month), OR = 0.54 (95% CI = 0.35 0.83). An inverse association was observed for women who used non aspirin NSAIDs for a duration of ⩾5 years, although not statistically significant (OR = 0.71, 95% CI = 0.47 1.07). In combined analyses of frequency and duration of use, inverse associations with EOC risk, although not statistically significant, were observed for all categories of aspirin use. However, a statistically significant lower risk of EOC was present among women who used non aspirin NSAIDs for <30 days per month for a duration of ⩾5 years (OR = 0.47, 95% CI = 0.28 0.79). Although inversely associated, no statistically significant associations were observed for frequency or duration of acetaminophen use.

The most common indication of aspirin use was heart disease prevention (75.3%), whereas arthritis (45.5%) was the most common reason for non aspirin NSAID use. Significant inverse associations were observed for the most prevalent indication of use for aspirin and non aspirin NSAIDs, where a 50% lower EOC risk was observed for women using aspirin to prevent heart disease (OR = 0.50, 95% CI = 0.29 0.85) and a 48% lower EOC risk for women using non aspirin NSAIDs for arthritis (OR = 0.52, 95% CI = 0.31 0.88) (data not shown).

The influence of dose for analgesic medications on EOC risk was also examined; however, sufficient data on dose were only present for aspirin use. Of the aspirin users with dosing information (n = 127), 77.2% reported low dose aspirin use. Low dose aspirin users had a more pronounced, statistically significant inverse association with EOC risk, OR = 0.54 (95% CI = 0.31 0.97), in comparison with high dose aspirin users, OR = 0.78 (95% CI = 0.30 2.06) (data not shown).

Presence of effect modification by BMI and smoking on the relationship between analgesic medications and EOC risk was not observed, P < 0.05 (data not shown).

DISCUSSION

This is the first study to examine the association between analgesic drug use and EOC risk in a large population of AA women. A statistically significant 44% lower EOC risk and a borderline significant 26% lower EOC risk was observed among aspirin and non aspirin NSAID users, respectively. Although inversely related, no significant associations for acetaminophen use and EOC risk were observed in this population. The inverse association between analgesic use and EOC was consistent with previous findings from several studies (Schildkraut *et al*, 2006; Wernli *et al*, 2008; Pinheiro *et al*, 2009), and especially parallel the findings in the recent pooled analysis (Trabert *et al*, 2014). As with the pooled analysis, a similar inverse association for aspirin use and EOC risk, overall, was observed and especially for low dose aspirin use, daily aspirin use, and shorter duration of aspirin use (<5 years). The present study also observed a decreased EOC risk for non aspirin NSAID use

Table 1. Characteristics[a] of cases and controls, AACES (*N* 1272)

	Cases (*n* 541)	Controls (*n* 731)	
	N (%)	*N* (%)	*P* value
Age, years[b]			
<50	124 (23.1)	198 (27.1)	0.02
50–59	188 (35.1)	272 (37.2)	
60–69	143 (26.7)	189 (25.8)	
70+	81 (15.1)	72 (9.9)	
Education			
⩽High school	242 (44.7)	271 (37.1)	0.02
Some post high school training	139 (25.7)	207 (28.3)	
College or graduate degree	160 (29.6)	253 (34.6)	
Income			
<$25 000	254 (47.9)	320 (44.3)	0.18
$25 000–$49 999	130 (24.5)	162 (22.4)	
$50 000–$74 999	76 (14.4)	121 (16.7)	
$75 000+	70 (13.2)	120 (16.6)	
Parity (number of live births)			
Nulliparous	105 (19.4)	93 (12.7)	0.01
1	101 (18.7)	137 (18.7)	
2	123 (22.7)	194 (26.6)	
3+	212 (39.2)	307 (42.0)	
Family history of breast or ovarian cancer			
No	383 (72.5)	577 (81.6)	0.0002
Yes	145 (27.5)	130 (18.4)	
Tubal ligation			
No	351 (64.9)	431 (59.0)	0.03
Yes	190 (35.1)	300 (41.0)	
BMI (kg m ^{2})			
<25 (underweight and normal weight)	79 (14.7)	136 (18.6)	0.16
25–29.9 (overweight)	139 (25.8)	189 (25.9)	
30+ (obese)	320 (59.5)	405 (55.5)	
Oral contraceptive use			
Never	163 (30.1)	151 (20.7)	0.0001
Ever	378 (69.9)	580 (79.3)	
Menopausal status			
Pre-menopausal	84 (15.6)	134 (18.4)	0.29
Peri-menopausal	73 (13.5)	84 (11.5)	
Post-menopausal	382 (70.9)	511 (70.1)	
Endometriosis			
No	476 (88.6)	696 (95.2)	0.00001
Yes	61 (11.4)	35 (4.8)	
Pelvic inflammatory disease			
No	491 (91.4)	692 (94.9)	0.01
Yes	46 (8.6)	37 (5.1)	
Mild intensity physical activity			
No	389 (72.3)	462 (63.2)	0.0007
Yes	149 (27.7)	269 (36.8)	
Moderate or strenuous intensity physical activity			
No	301 (55.7)	426 (58.3)	0.37
Yes	239 (44.3)	305 (41.7)	
Histologic subtype			
Serous	367 (72.8)		
Mucinous	24 (4.7)		
Endometrioid	70 (13.9)		
Clear cell	12 (2.4)		
Mixed	14 (2.8)		
Other	17 (3.4)		

Abbreviations: AACES African American Cancer Epidemiology Study; BMI body mass index.
[a]Missing data on age for 5 women, income for 19 women, family history of a first-degree relative with breast or ovarian cancer for 37 women, BMI for 4 women, menopausal status for 4 women, endometriosis for 4 women, pelvic inflammatory disease for 6 women, mild intensity physical activity for 3 women, and moderate or strenuous intensity physical activity for 1 woman, and 37 cases for histologic subtype.
[b]Age at diagnosis for cases and age at the time of interview for controls.

overall and specifically for occasional monthly use (<30 days per month) for ⩾5 years. The previously published pooled analysis only observed a significant inverse association for high dose non aspirin NSAID use (Trabert *et al*, 2014); however, the non aspirin NSAID use results of the present study were similar to many published studies (Fairfield *et al*, 2002; Schildkraut *et al*, 2006; Merritt *et al*, 2008; Wernli *et al*, 2008; Pinheiro *et al*, 2009).

Interestingly, the inverse relationship between analgesic use and EOC risk observed in the present study was more pronounced than those observed in predominately white populations. In the only other study to conduct race specific analyses for analgesic use and EOC risk, AA women had the strongest inverse association between aspirin and non aspirin NSAID use and EOC risk, although not significant (Setiawan *et al*, 2012). Similar differences in the magnitude of effect by race have been reported for breast cancer, where use of analgesic medications was associated with a stronger inverse relationship for risk of breast cancer among AA women compared with all other races (Gill *et al*, 2007). As AA women have higher levels of inflammation, in general, compared with white women (Albert *et al*, 2004; Khera *et al*, 2005; Paalani *et al*, 2011), the potential benefit of taking anti inflammatory drugs may be greater among AA women. The observed racial differences may also be because of a variety of factors that could vary by race, including genetic variants (e.g., prevalence of polymorphisms affecting cyclooxygenase activity), cultural attitudes toward analgesic use, or patterns of analgesic use. Further research needs to be conducted among racially diverse populations to confirm the observed racial differences in effect.

Although our findings suggest a protective effect for aspirin taken daily, at low doses, and for heart disease prevention, these three characteristics of use are highly correlated. A daily, low dose of aspirin is typically recommended for women at high risk of a cardiovascular event. In the WaTCH study, of the women who took aspirin for heart disease prevention, >85% also reported low dose aspirin use and a daily frequency of use. As the overwhelming majority of women taking aspirin for heart disease are the same women using aspirin daily and at low doses, it is difficult to determine whether indication, frequency, dose, or a combination of these characteristics of use is contributing to the reduction in risk. In addition, the observed association between aspirin use for heart disease prevention and EOC risk in the present study may be explained by the effects of other cardiovascular drugs (e.g., β blockers, statins, angiotensin converting enzyme inhibitors) taken concurrently with analgesic medications. To explore this possibility, the cardiovascular medication use of women taking aspirin for heart disease prevention ($n=122$) was examined; although a slightly higher prevalence of these cardiovascular medications was taken by the controls, a statistically significant difference in drug use was not observed (data not shown).

Although ovarian cancer is associated with a long latency period, our findings suggest that shorter durations of aspirin use (<5 years) confer a stronger protective effect. Previous literature evaluating this relationship is inconsistent; some studies (Akhmedkhanov *et al*, 2001; Wernli *et al*, 2008), including the large pooled analysis (Trabert *et al*, 2014), observed a stronger inverse association for shorter durations of aspirin use, whereas other studies reported a stronger protection for longer durations of use (Rosenberg *et al*, 2000; Lacey *et al*, 2004; Schildkraut *et al*, 2006). Although unclear, we speculate that the findings for shorter durations of use could be because of a few factors. A recall bias may be present, resulting in misclassification of duration. Women may have had difficulty accurately recalling their duration of use if it occurred for a longer period of time. In addition, as analgesic medications are routinely used for a variety of indications, women may not keep track of each use, leading to an underestimation of duration. The majority of women reporting a short duration of aspirin use began taking these medications within 2 5 years of their diagnosis (cases) or the time of interview (controls). It is

possible that using aspirin during this time period may be more beneficial in protecting against ovarian cancer progression. In fact, cyclooxygenase inhibitors (i.e., aspirin) have been shown to decrease cell growth, increase apoptosis, and block angiogenesis (Dubois *et al*, 1998). Experimental studies are needed to explore a mechanistic explanation as to why a shorter duration of aspirin use may provide a greater protection against ovarian cancer.

The present study has several strengths. First, data from the largest study of EOC among AA women were utilised, resulting in a relatively large sample size to examine race specific associations for analgesic use. Another strength is the detailed exposure assessment of analgesic use, allowing for effect estimates by frequency, duration, and indication of use. Despite these strengths, there were several limitations present in this study. Data on analgesic drug use were ascertained through self report and may be subject to potential biases. In particular, if participants used several different types of these medications during their lifetime, it may be difficult to accurately report their use of each, especially with regard to the duration, frequency, and indication of use. However, the associations observed in the present study are consistent with those from the large pooled analysis of the Ovarian Cancer Association Consortium (Trabert *et al*, 2014) and other cohort studies (Setiawan *et al*, 2012), although not significant. In addition, women with cardiovascular disease may be more acutely aware of their aspirin use; however, the prevalence of heart disease is similar among cases and controls (~11%) and any resulting misclassification would bias the effect estimates towards the null. The lack of information on dose for non aspirin NSAIDs and acetaminophen use was another limitation in this study. Because of the case control study design, a potential selection bias may be present (i.e., women using analgesic medications may be more healthy and more likely to participate in the study). To evaluate the potential for a selection bias, the prevalence of analgesic use among AACES controls was compared with the available estimates for the general US population (Zhou *et al*, 2014). Although a direct comparison group for the demographic of the AACES study was unavailable, the prevalence of aspirin use among Black adults, aged ⩾18 years, was similar to AACES controls, and although possible, a selection bias is unlikely. Finally, although AACES is the largest study of EOC in AA, the sample of AA women within certain categories of exposure (e.g., indication of aspirin use, combined frequency, and duration of use) was small, limiting the power and precision of the effect estimates.

Table 2. Estimated ORs and 95% CIs for the association of analgesic medication use and ovarian cancer risk

Analgesic medication use			
	No. of cases (*n* = 541)	**No. of controls (*n* = 731)**	**Adjusted OR[a] (95% CI)**
Never users	362	467	1.00 (Referent)
Any NSAID[b] only	125	197	0.73 (0.54–0.98)
Acetaminophen only	23	33	0.98 (0.53–1.81)
Both NSAIDs and acetaminophen	31	34	1.03 (0.58–1.84)
Aspirin use			
	No. of cases (*n* = 432)	**No. of controls (*n* = 560)**	**Adjusted OR[a,c] (95% CI)**
Never users	362	467	1.00 (Referent)
Ever use	70	93	0.56 (0.35–0.92)
Non aspirin NSAID use			
	No. of cases (*n* = 474)	**No. of controls (*n* = 628)**	**Adjusted OR[a,c] (95% CI)**
Never users	362	467	1.00 (Referent)
Ever use	112	161	0.74 (0.52–1.05)
Acetaminophen use			
	No. of cases (*n* = 416)	**No. of controls (*n* = 534)**	**Adjusted OR[a,c] (95% CI)**
Never users	362	467	1.00 (Referent)
Ever use	54	67	0.89 (0.49–1.62)

Abbreviations: CI confidence interval; NSAID nonsteroidal anti-inflammatory drug; OR odds ratio.
[a]Adjusted for age, study site, education, income, parity, family history of first-degree relative with breast or ovarian cancer, tubal ligation, body mass index (BMI), oral contraceptive use, menopausal status, endometriosis, pelvic inflammatory disease, and physical activity.
[b]Any NSAID use includes aspirin and non-aspirin NSAIDs, but not acetaminophen.
[c]Also simultaneously adjusted for use of other analgesics.

Table 3. Estimated ORs and 95% CIs for the association of frequency and duration of aspirin, non-aspirin NSAIDs, and acetaminophen use with ovarian cancer risk

	Aspirin			**Non aspirin NSAID**			**Acetaminophen**		
	No. of cases (*n* 432)	**No. of controls (*n* 560)**	**Adjusted OR[a] (95% CI)**	**No. of cases (*n* 474)**	**No. of controls (*n* 628)**	**Adjusted OR[a] (95% CI)**	**No. of cases (*n* 416)**	**No. of controls (*n* 534)**	**Adjusted OR[a] (95% CI)**
Never users	362	467	1.00 (Referent)	362	467	1.00 (Referent)	362	467	1.00 (Referent)
Frequency of use									
<30 Days/month	15	27	0.51 (0.21–1.25)	58	108	0.54 (0.35–0.83)	35	48	0.94 (0.49–1.81)
Daily	55	66	0.56 (0.34–0.94)	54	53	1.15 (0.70–1.91)	19	19	0.79 (0.33–1.92)
Duration of use									
<5 Years	27	41	0.52 (0.28–0.98)	33	51	0.81 (0.48–1.37)	15	25	0.97 (0.42–2.22)
⩾5 Years	43	52	0.60 (0.32–1.11)	79	110	0.71 (0.47–1.07)	39	42	0.87 (0.44–1.74)
Frequency, duration of use									
<30 Days/month for <5 years	7	13	0.43 (0.14–1.34)	20	33	0.67 (0.35–1.27)	8	18	0.94 (0.34–2.63)
Daily for <5 years	20	28	0.56 (0.27–1.11)	13	18	1.13 (0.48–2.66)	7	7	1.12 (0.32–3.96)
<30 Days/month for ⩾5 years	8	14	0.61 (0.17–2.22)	38	75	0.47 (0.28–0.79)	27	30	1.00 (0.45–2.21)
Daily for ⩾5 years	35	38	0.56 (0.29–1.08)	41	35	1.17 (0.65–2.09)	12	12	0.58 (0.19–1.79)

Abbreviations: CI confidence interval; NSAID nonsteroidal anti-inflammatory drug; OR odds ratio.
[a]Adjusted for age, study site, education, income, parity, family history of first-degree relative with breast or ovarian cancer, tubal ligation, body mass index (BMI), oral contraceptive use, menopausal status, endometriosis, pelvic inflammatory disease, physical activity, and simultaneous adjustment of use of other analgesics (frequency, duration).

In conclusion, this study supports previous evidence that any NSAID use, but not acetaminophen, is inversely associated with EOC risk. The findings of the present study raise the intriguing possibility that the inverse association may be stronger in AA women compared with white women. This possibility emphasises the value of studying this question among racially diverse populations. Future research, specifically in large cohort studies, is needed in order to fully elucidate the impact of analgesic drug use on EOC risk in AA women, as well as other underrepresented racial groups.

ACKNOWLEDGEMENTS

This study was supported by the National Cancer Institute (Grant CA142081). Additional support was provided by the Metropolitan Detroit Cancer Surveillance System with funding from the National Cancer Institute, National Institute of Health, the Department of Health and Human Services (Contract HHSN261201000028C), and the Epidemiology Research Core, supported in part by the National Cancer Institute Center (Grant P30 CA22453) to the Karmanos Cancer Institute, Wayne State University School of Medicine). We are grateful to the AACES interviewers, Christine Bard, LaTonda Briggs, Whitney Franz (North Carolina), and Robin Gold (Detroit). We also acknowledge the individuals responsible for facilitating case ascertainment across all AACES sites.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Exhibit 109

American Journal of Epidemiology

Vol. 185, No. 1
DOI: 10.1093/aje/kww161
Advance Access publication:
December 9, 2016

Systematic Reviews and Meta- and Pooled Analyses

Pelvic Inflammatory Disease and the Risk of Ovarian Cancer and Borderline Ovarian Tumors: A Pooled Analysis of 13 Case-Control Studies

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Initially submitted November 17, 2015; accepted for publication May 26, 2016.

Inflammation has been implicated in ovarian carcinogenesis. However, studies investigating the association between pelvic inflammatory disease (PID) and ovarian cancer risk are few and inconsistent. We investigated the association between PID and the risk of epithelial ovarian cancer according to tumor behavior and histotype. We pooled data from 13 case-control studies, conducted between 1989 and 2009, from the Ovarian Cancer Association Consortium (OCAC), including 9,162 women with ovarian cancers, 2,354 women with borderline tumors, and 14,736 control participants. Study-specific odds ratios were estimated and subsequently combined into a pooled odds ratio using a random-effects model. A history of PID was associated with an increased risk of borderline tumors (pooled odds ratio (pOR) = 1.32, 95% confidence interval (CI): 1.10, 1.58). Women with at least 2 episodes of PID had a 2-fold increased risk of borderline tumors (pOR = 2.14, 95% CI: 1.08, 4.24). No association was observed between PID and ovarian cancer risk overall (pOR = 0.99, 95% CI: 0.83, 1.19); however, a statistically nonsignificantly increased risk of low-grade serous tumors (pOR = 1.48, 95% CI: 0.92, 2.38) was noted. In conclusion, PID was associated with an increased risk of borderline ovarian tumors, particularly among women who had had multiple episodes of PID. Although our results indicated a histotype-specific association with PID, the association of PID with ovarian cancer risk is still somewhat uncertain and requires further investigation.

inflammation; neoplasms; histological type; ovarian neoplasms; pelvic inflammatory disease; risk factors

Abbreviations: AUS, Australian Ovarian Cancer Study/Australian Cancer Study (Ovarian Cancer); CI, confidence interval; CON, Connecticut Ovarian Cancer Study; DOV, Diseases of the Ovary and Their Evaluation; HAW, Hawaii Ovarian Cancer Study; HOP, Hormones and Ovarian Cancer Prediction; MAL, Danish Malignant Ovarian Tumor Study; NCO, North Carolina Ovarian Cancer Study; NJO, New Jersey Ovarian Cancer Study; NTH, Nijmegen Polygene Study and Nijmegen Biomedical Study; OCAC, Ovarian Cancer Association Consortium; OR, odds ratio; PID, pelvic inflammatory disease; pOR, pooled odds ratio; SON, Southern Ontario Ovarian Cancer Study; TOR, Familial Ovarian Tumor Study; UCI, University of California Irvine Ovarian Cancer Study; USC, Los Angeles County Case-Control Studies of Ovarian Cancer.

Ovarian cancer is the fifth most common cancer among women in developed countries, and it is the most fatal gynecological malignancy (1). The etiology of ovarian cancer is still not fully clarified, although a number of risk factors have been identified. A reduced risk of ovarian cancer has been observed with increased parity (2), use of oral contraceptives (2), hysterectomy (3), and tubal ligation (3), whereas family history of ovarian or breast cancer (2), use

of hormone replacement therapy (2), exposure to talc (4), and a history of endometriosis (5) have been associated with increased risks.

The 2 dominant hypotheses to explain the development of ovarian cancer relate increased risk to a large number of lifetime ovulatory cycles (the incessant ovulation theory) (6) or exposure to high levels of gonadotropins (the gonadotropin theory) (7). However, inflammation has also been suggested as a potential biological mechanism that may underlie a number of epidemiologic associations not easily explained by either theory (8, 9), including talc exposure, endometriosis, tubal ligation, and hysterectomy. Furthermore, a link between pelvic inflammatory disease (PID) and the risk of ovarian cancer has been suggested, and this potential association may also be explained by the inflammation theory. PID is defined as an upper genital-tract infection and includes diagnoses of endometritis, salpingitis, pelvic peritonitis, and tubo-ovarian abscess caused by microorganisms ascending from the lower genital tract (10). Approximately 800,000 women are treated for PID annually in the United States (11), and it is estimated that 6% 20% of all women in the Western world are diagnosed with PID during their lifetimes (12 14).

Epidemiologic studies investigating the association between PID and the risk of ovarian cancer and borderline ovarian tumors have been inconsistent, revealing increased risks in some studies (15 19) but not in all (20 23). Moreover, most previous studies have had methodological problems, including limited statistical power due to small numbers of study subjects and/or a short follow-up period. Also, ovarian cancer is a heterogeneous disease consisting of different histotypes with different risk factor profiles (24). However, few investigators have studied the role of PID separately for borderline tumors (15, 18) or for the separate histotypes of ovarian cancer (18, 20).

To examine the association of PID with the risk of ovarian cancer, an international collaborative study was performed, using data from 13 case-control studies participating in the Ovarian Cancer Association Consortium (OCAC). To our knowledge, this was the largest study of PID and ovarian cancer risk to date, thereby enabling a more robust estimation of risks among subgroups according to tumor behavior and histotype than has previously been possible.

METHODS

Participating studies

OCAC was founded in 2005 as an international forum of investigators conducting ovarian cancer case-control studies. The main aims of the collaboration are to discover associations between genetic polymorphisms and ovarian cancer risk and to identify and confirm epidemiologic risk factors for ovarian cancer (25).

For the present study, we obtained individual-level data from 13 case-control studies: 12 studies in OCAC (20, 26 37) and a parallel study not originally included in OCAC (Southern Ontario Ovarian Cancer Study (SON)) (38). Eight studies were conducted in the United States (Connecticut Ovary Study (CON), Diseases of the Ovary and Their Evaluation (DOV), Hawaii Ovarian Cancer Study (HAW), Hormones and Ovarian Cancer Prediction (HOP), North Carolina Ovarian Cancer Study (NCO), New Jersey Ovarian Cancer Study (NJO), University of California Irvine Ovarian Cancer Study (UCI), and Los Angeles County Case-Control Studies of Ovarian Cancer (USC)) (26, 27, 31 36), 2 in Canada (Familial Ovarian Tumor Study (TOR) and SON) (37, 38), 2 in Europe (Danish Malignant Ovarian Tumor Study (MAL) and Nijmegen Polygene Study and Nijmegen Biomedical Study (NTH)) (28 30), and 1 in Australia (Australian Ovarian Cancer Study and Australian Cancer Study (Ovarian Cancer) (AUS)) (20).

Characteristics of the 13 included studies are presented in Table 1. Data were cleaned and checked for internal consistency, and clarifications were obtained from the initial investigators if needed. Women with nonepithelial ovarian tumors ($n = 186$) and with missing information on PID status ($n = 278$) were excluded, leaving 9,162 women with invasive ovarian cancer (hereafter denoted "ovarian cancer"), 2,354 women with borderline ovarian tumors, and 14,736 control participants for analysis. Eleven studies included both women with ovarian cancer and women with borderline ovarian tumors, whereas 2 studies included only women with ovarian cancer (NTH and NJO). Each study had approval from the relevant institutional review board or ethics committee, and all participants gave informed consent.

PID assessment

Information on PID was self-reported in all studies, through either in-person interviews ($n = 10$ studies) or self-administered questionnaires ($n = 3$ studies). Table 1 includes the phrasing of the question regarding PID status used in each study. We aimed to obtain information on the following PID variables: PID status (ever/never had PID), age at first PID episode, time since first PID episode, and number of PID episodes. All studies except for HAW had information on age at first PID episode, and 5 studies (CON, DOV, NJO, SON, and TOR) had data on number of PID episodes.

Statistical analysis

Associations between the PID variables and ovarian cancer risk were estimated using a 2-stage method (39). First, study-specific odds ratios were obtained from logistic regression models and were subsequently combined into a pooled odds ratio with 95% confidence intervals. The pooled estimate was computed by weighting each estimate by the inverse of the sum of its variance and the across-studies variance using a random-effects model (40). Only studies for which the study-specific model converged contributed to the pooled estimate. We used the Cochran Q and I^2 statistics to evaluate statistical heterogeneity between studies. If heterogeneity was present, we explored the potential sources of heterogeneity, including continent of study (North America vs. Europe vs. Australia) and method of data collection (in-person interview vs. self-administered questionnaire).

Table 1. Characteristics of 13 Ovarian Cancer Case-Control Studies From the Ovarian Cancer Association Consortium, Conducted in Australia, Europe, and North America Between 1989 and 2009

First Author, Year (Reference No.)	Study Name and Acronym	Study Period	Study Type	Method of Data Collection	Age Range, years	Matching Variable	Mean Interval From Ovarian Cancer to Interview, months	Response Rate, %		Wording of Question Concerning PID Status	No. and % of Controls Who Had Had PID		Missing PID Data
								Cases	Controls		No.	%	%
Australia													
Merritt, 2008 (20)	Australian Ovarian Cancer Study/ Australian Cancer Study (Ovarian Cancer) (AUS)	2002–2005	Population-based	Self-administered questionnaire	18–80	Age (5-year categories)	5.3	84	47	Have you ever had pelvic inflammatory disease (e.g., chlamydia)? Have you ever had infection of the tubes or womb?	84	5.6	3.5
Europe													
Glud, 2004 (28)	Danish Malignant Ovarian Tumor Study (MAL)	1995–1999	Population-based	In-person interview	31–81	Age (5-year categories)	3.6	81	68	Have you ever been told by a doctor that you had pelvic inflammatory disease, that is an infection in your uterus or tubes?[a]	416	26.6	0.7
van Altena, 2012 (29) Wetzels, 2007 (30)	Nijmegen Polygene Study and Nijmegen Biomedical Study (NTH)	1989–2008	Population-based	Self-administered questionnaire	23–83	No matching	85.3	63	42	Could you tell whether you have ever had inflammation of the tubes or ovaries?	13	2.2	0.0
North America													
Risch, 2006 (34)	Connecticut Ovarian Cancer Study (CON)	1998–2003	Population-based	In-person interview	34–81	Age (3 age groups: 35–49 years, 50–64 years, and 65–79 years)	9.6	69	61	Could you tell me whether you have ever had an internal pelvic infection, sometimes called PID or pelvic inflammatory disease? We are not including bladder or vaginal infections in this.	23	4.2	0.2
Bodelon, 2012 (27)	Diseases of the Ovary and Their Evaluation (DOV)	2002–2009	Population-based	In-person interview	35–74	Age (5-year categories)	9.3	74	62	Before reference date, did a doctor or other health professional ever tell you that you had pelvic inflammatory disease or PID?[a]	65	3.5	0.3
Goodman, 2008 (31)	Hawaii Ovarian Cancer Study (HAW)	1993–2008	Population-based	In-person interview	18–93	Age (5-year categories), race/ ethnicity	10.9	78	80	Have you ever had PID or pelvic inflammatory disease? That is, have you ever had an infection in your tubes?	27	2.5	0.0

Table continues

Table 1. Continued

First Author, Year (Reference No.)	Study Name and Acronym	Study Period	Study Type	Method of Data Collection	Age Range, years	Matching Variable	Mean Interval From Ovarian Cancer to Interview, months	Response Rate, %		Wording of Question Concerning PID Status	No. and % of Controls Who Had Had PID		Missing PID Data
								Cases	Controls		No.	%	%
Lo-Ciganic, 2012 (32)	Hormones and Ovarian Cancer Prediction (HOP)	2003–2009	Population-based	In-person interview	25–94	Age (5-year categories)	4.3	71	68	Before reference date, did a doctor or other health professional ever tell you that you had pelvic inflammatory disease (PID) or pelvic infection not related to surgery?[a]	22	1.2	0.0
Schildkraut, 2010 (35)	North Carolina Ovarian Cancer Study (NCO)	1999–2008	Population-based	In-person interview	20–75	Age (5-year categories), race/ethnicity	6.2	67	60	Before you were diagnosed with ovarian cancer, had a doctor ever told you that you had pelvic inflammatory disease (or other pelvic infection)?[a]	37	3.4	0.3
Bandera, 2011 (26)	New Jersey Ovarian Cancer Study (NJO)	2002–2008	Population-based	In-person interview	23–88	No matching	11.4	47	40	Before reference date, were you ever told by a health professional that you had PID or pelvic inflammatory disease?[a]	2	0.4	0.9
Risch, 1994 (38)	Southern Ontario Ovarian Cancer Study (SON)	1989–1992	Population-based	In-person interview	25–80	Age (3 age groups: 35–49 years, 50–64 years, and 65–79 years)	4.8	71	65	Could you tell me whether you have ever had an internal pelvic infection? (PID or pelvic inflammatory disease—not including your bladder or vagina)	114	20.2	1.2
Zhang, 2011 (37)	Familial Ovarian Tumor Study (TOR)	1995–2003	Population-based[b]	In-person interview	21–94	Age (5-year categories)	21.4	50	80	Could you tell me whether you have ever had an internal pelvic infection, sometimes called PID or pelvic inflammatory disease? We are not including bladder or vaginal infections in this.	14	2.6	0.0
Ziogas, 2000 (36)	University of California Irvine Ovarian Cancer Study (UCI)	1993–2005	Population-based	Self-administered questionnaire	18–86	Age (5-year categories), race/ethnicity	31.6	65	80	Have you ever been told by a physician that you have pelvic inflammatory disease?[a]	28	4.6	8.9

Table continues

Table 1. Cont nued

First Author, Year (Reference No.)	Study Name and Acronym	Study Period	Study Type	Method of Data Collection	Age Range, years	Matching Variable	Mean Interval From Ovarian Cancer to Inter view, months	Response Rate, %		Wording of Question Concerning PID Status	No. and % of Controls Who Had Had PID		Missing PID Data
								Cases	Controls		No.	%	%
P ke, 2004 (33)	Los Ange es County Case-Contro Stud es of Ovar an Cancer (USC)	1992–2009	Popu at on-based	In-person nterv ew	19–86	Age (5-year categor es), race/ ethn c ty	8.1	73	73	Have you ever had PID or pe v c nflammatory d sease? That s, have you ever had an nfect on n your tubes? Before [month/year], d d a doctor ever te you that you had PID or pe v c nflammatory d sease?[b]	99	3.8	0.2

Abbrev at on: PID, pe v c nflammatory d sease.
[a] Stud es c ass fied as hav ng a requ rement that the d agnos s of PID be ver fied by a phys c an.
[b] Popu at on-based cases and non–popu at on-based contro s.

For analyses, age at first PID episode and time since first PID episode were modeled both as categorical and continuous variables. Each categorical variable was categorized into ordinal groups (age at first PID episode: <20, 20 29, or ≥30 years; time since first PID episode: <10, 10 19, or ≥20 years; number of PID episodes: 1 or ≥2), with women who had never had PID as the referent. Associations between the continuous variables (age at first PID episode and time since first PID episode) and ovarian cancer risk were assessed only among women who had ever been diagnosed with PID. In order to model these associations, we included PID status in the model as a categorical indicator variable together with the continuous PID variable, as suggested by Leffondré et al. (41).

All analyses adjusted for age, parity (nulliparous vs. parous as well as parity as a continuous variable), oral contraceptive use (ever/never use as well as duration of use as a continuous variable), and family history of ovarian or breast cancer in a first-degree relative (yes/no) irrespective of their effect on the association between PID and ovarian cancer risk, because these factors were considered to be potentially important confounders a priori. For studies that used matching (age, race/ethnicity), conditional logistic regression analysis was used to adjust for these variables. In unmatched studies, age was categorized into 5-year age groups and unconditional logistic regression analysis was used (Table 1). When modeling parity and oral contraceptive use, the categorical variable was included as an indicator variable together with the continuous variable (41). Other potential confounders were considered but were not included in the final model, because none of them fulfilled an inclusion criterion of changing the log of the pooled estimate for ovarian cancer risk by 10% or more; these potential confounders were tubal ligation, hysterectomy, endometriosis, use of hormone replacement therapy, breastfeeding, age at menarche, menopausal status, body mass index, cigarette smoking, and educational level.

We examined interactions between PID status and parity (nulliparous vs. parous), oral contraceptive use (ever use vs. never use), and family history of ovarian or breast cancer in first-degree relatives (yes vs. no). Family history of breast or ovarian cancer was used as a proxy for hereditary ovarian cancer, as we aimed at exploring whether PID was similarly associated with hereditary and sporadic ovarian cancer. Linearity for all quantitative variables was examined by comparison with models with restricted cubic splines, but no appreciable deviations from linearity were found. The significances of the interactions and nonlinear associations were estimated by likelihood ratio tests of the interactions/nonlinearities and then comparison of the distribution of the study-specific P values with a uniform distribution by means of the Kolmogorov-Smirnov test (42).

All analyses were performed separately for ovarian cancer and for borderline tumors, and subgroup analyses were conducted by histotype. Ovarian cancers were divided into categories of serous, mucinous, endometrioid, clear cell, and other (including mixed cell, undifferentiated, and tumors of unknown epithelial histology). Additionally, serous cancers were divided into low-grade (grade 1) and high-grade

(grade 2 or higher) tumors, because these are considered to represent different histotypes (43). However, 2 studies had no information on grade (SON and TOR) and were therefore not included in these analyses; they were included only in the analyses for serous cancer overall. Subgroup analyses for borderline ovarian tumors included serous and mucinous tumors, because other histotypes of borderline ovarian tumors are rare. All *P* values were 2-sided, and the nominal level of statistical significance was set at $P < 0.05$. All statistical analyses were performed using the statistical software R, version 3.1.2 (R Foundation for Statistical Computing, Vienna, Austria), including the packages "survival," "meta," and "rms."

RESULTS

A history of PID was reported by 500 of the 9,162 women with ovarian cancers (5.5%), by 201 of the 2,354 women with borderline ovarian tumors (8.5%), and by 944 of the 14,736 control participants (6.4%). The proportion of control participants with PID varied across study sites, from 0.4% to 26.6%. In 11 of the studies, small proportions (less than 6%) of control participants reported PID, whereas in a Canadian study (SON) and in the Danish study (MAL), larger proportions of the control participants reported having had PID (20.2% and 26.6%, respectively). Median age at first PID episode was 28 years (interquartile range, 22 36 years) among women with ovarian cancer, 24 years (interquartile range, 20 30 years) among women with borderline ovarian tumors, and 25 years (interquartile range, 20 33 years) among control participants. Distributions of the various histotypes of ovarian tumors from the included studies are provided in Web Table 1 (available at http://aje.oxfordjournals.org/).

Ovarian cancer

In the pooled analysis, we found no association between a history of PID and the risk of ovarian cancer (odds ratio (OR) = 0.99, 95% confidence interval (CI): 0.83, 1.19) (Web Table 2 and Figure 1). Furthermore, we observed no convincing associations of the age at first PID episode, time since first PID episode, or number of PID episodes with the risk of ovarian cancer (Web Table 2).

The magnitudes of the risk estimates for associations of specific histotypes of ovarian cancer with the individual

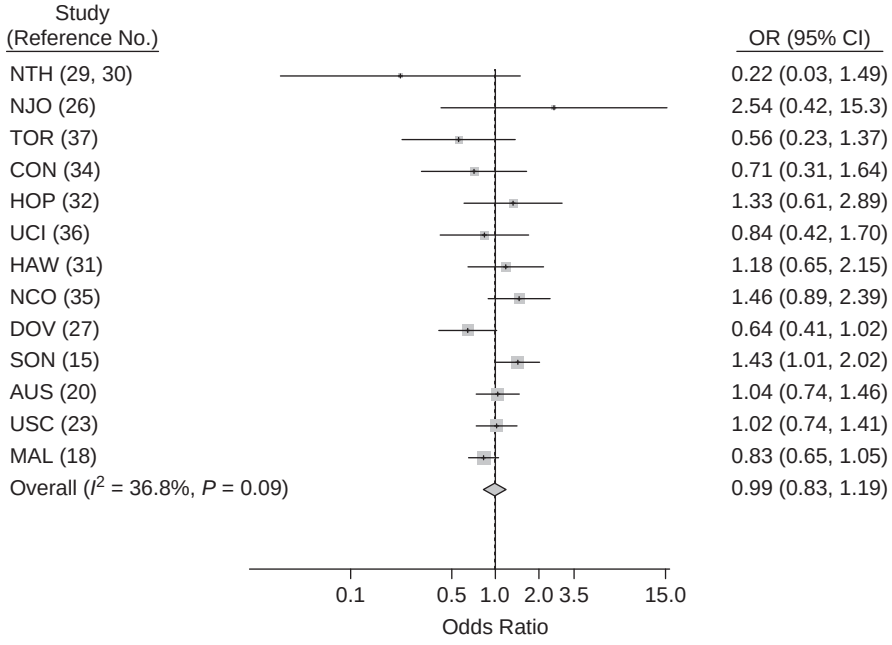

Figure 1. Associations between pelvic inflammatory disease (PID) status and the risk of ovarian cancer among the participants of 13 case-control studies in Australia, Europe, and North America, conducted between 1989 and 2009. Results are presented according to study site and overall and are adjusted for age, parity, oral contraceptive use (ever/never use and duration of use), and family history of ovarian or breast cancer (yes/no). For 4 of the studies (AUS, MAL, SON, and USC), results for the association between PID and ovarian cancer risk have been published previously (15, 18, 20, 23). For the remaining 9 studies, results for the association between PID and ovarian cancer risk have not been published previously, and their references therefore refer to papers with general information about these studies (26, 27, 29–32, 34–37). For the present study, we obtained individual-level data from all 13 studies directly from the Ovarian Cancer Association Consortium database. Each square and line represent the odds ratio (OR) and 95% confidence interval (CI), respectively, and the size of the square indicates the study weighting. AUS, Australian Ovarian Cancer Study and Australian Cancer Study (Ovarian Cancer); CON, Connecticut Ovarian Cancer Study; DOV, Diseases of the Ovary and Their Evaluation; HAW, Hawaii Ovarian Cancer Study; HOP, Hormones and Ovarian Cancer Prediction; MAL, Danish Malignant Ovarian Tumor Study; NCO, North Carolina Ovarian Cancer Study; NJO, New Jersey Ovarian Cancer Study; NTH, Nijmegen Polygene Study and Nijmegen Biomedical Study; SON, Southern Ontario Ovarian Cancer Study; TOR, Familial Ovarian Tumor Study; UCI, University of California Irvine Ovarian Cancer Study; USC, Los Angeles County Case-Control Studies of Ovarian Cancer.

PID variables did not differ from those observed for ovarian cancer overall, and only a few of the risk estimates reached statistical significance. However, we noted a higher risk of low-grade serous cancer (OR = 1.48, 95% CI: 0.92, 2.38) associated with PID status, although the risk estimate did not reach statistical significance (Web Table 2).

Borderline ovarian tumors

A history of PID was associated with a higher risk of borderline ovarian tumors (OR = 1.32, 95% CI: 1.10, 1.58) (Table 2 and Figure 2). Furthermore, women with 2 or more episodes of PID had a more than 2-fold higher risk of borderline ovarian tumors compared with women without a history of PID (OR = 2.14, 95% CI: 1.08, 4.24). We found no consistent trend in the risk of borderline tumors with age at first episode of PID (P-trend = 0.29) or time since first episode of PID (P-trend = 0.44).

As for borderline ovarian tumors overall, the risk of serous borderline ovarian tumors was statistically significantly increased among women with PID (OR = 1.43, 95% CI: 1.14, 1.79). Similarly, PID was also associated with an increased risk of mucinous borderline ovarian tumors, although the risk estimate was not statistically significant (OR = 1.28, 95% CI: 0.97, 1.68). The risks of serous and mucinous borderline ovarian tumors were not convincingly associated with age at or time since first PID episode. In addition, women with multiple episodes of PID had a higher risk of both serous and mucinous borderline ovarian tumors, but none of the risk estimates reached statistical significance (Table 2).

Additional analyses

To consider the possibility that early cancer symptoms might have been misinterpreted as PID or that an episode of PID might have resulted in further examinations that led to the identification of ovarian cancer, we performed sensitivity analyses of the association between PID status and the risk of ovarian cancer and borderline ovarian tumors by excluding women whose last PID episode was ≤1, ≤2, or ≤3 years before the date of diagnosis of ovarian cancer (for cases) or date of interview (for controls). The risk estimates in these sensitivity analyses were not substantially different from the risk estimates in the main analyses (data not shown).

We performed additional sensitivity analyses by stratifying studies by data collection method (in-person interview vs. self-administered questionnaire), study continent (North America vs. Europe vs. Australia), whether a physician-verified diagnosis of PID was required, study period (before or including 2000 vs. after 2000), proportion of control participants with PID (low (<6%) vs. high (>20%)), body mass index (calculated as weight (kg)/height (m)^2; <25 vs. ≥25), age at diagnosis of ovarian cancer (cases) or interview (controls) (<50 years vs. ≥50 years), and level of education (high school or less vs. more than high school). However, in the vast majority of these analyses, the direction and the magnitude of the associations were virtually unchanged compared with the associations obtained in the main analyses (data not shown). Notable exceptions were the observation of apparently statistically significantly increased risks of low-grade serous ovarian cancer (OR = 2.36, 95% CI: 1.24, 4.48) and endometrioid ovarian cancer (OR = 1.42, 95% CI: 1.01, 1.98) among women in the North American studies. However, no associations between PID and these 2 tumor types were found among the European studies or in the Australian study (low-grade serous cancer: pooled OR = 0.98, 95% CI: 0.61, 1.59 for the European studies and OR = 1.49, 95% CI: 0.52, 4.30 for the Australian study; endometrioid ovarian cancer: pooled OR = 0.60, 95% CI: 0.33, 1.10 for the European studies and OR = 1.09, 95% CI: 0.52, 2.26 for the Australian study).

Statistically significant heterogeneity across studies was observed for only a few of the risk estimates (Web Table 2 and Table 2). However, additional analyses showed that neither the method of data collection nor study continent nor proportion of control participants with PID could explain the observed heterogeneity since these additional analyses did not reveal increased consistency among studies of the same type (data not shown). We observed no effect modification between PID status and any of the potential risk factors (parity, oral contraceptive use, and family history of ovarian/breast cancer) for ovarian cancer and borderline ovarian tumors (all P values > 0.05) (data not shown).

DISCUSSION

To our knowledge, this was the largest study to date to have investigated the association between history of PID and the risk of ovarian cancer. In a pooled analysis of 13 case-control studies, we found no convincing associations between self-reported PID status and the risk of ovarian cancer overall, but suggestions of an increased risk of low-grade serous cancer were noted. For borderline ovarian tumors, an increased risk was observed among women with a history of PID, both overall and for serous and mucinous borderline tumors separately. Furthermore, the risk of borderline tumors increased with the number of PID episodes.

An association between PID and the risk of ovarian tumors is biologically plausible and could be explained by the inflammation hypothesis (8). Inflammation is characterized by the production of free radicals, cytokines, prostaglandins, and growth factors with the potential for genetic and epigenetic changes to the DNA, resulting in an increased risk of malignant transformation (44). Until recently, it was believed that all histotypes of ovarian cancer arose from the mesodermal surface epithelium, either on peritoneal surfaces or entrapped within the ovaries, and inflammation of the epithelium was therefore proposed to trigger malignant transformation (8). Recently, it has been suggested that some serous ovarian tumors originate in the mucosal epithelium of the fallopian tube, and inflammation of the fallopian tubes has been proposed to contribute to the development of these tumors (45).

The association between PID and the risk of ovarian cancer has been investigated in only 2 cohort studies (17, 19) and 7 case-control studies (15, 16, 18, 20–23). However, 4 of those case-control studies were based on data from study

Table 2. Adjusted Pooled Odds Ratios for the Association Between Pelvic Inflammatory Disease and Borderline Ovarian Tumors Among Participants in the Ovarian Cancer Association Consortium (Australia, Europe, and North America), 1989–2009

PID History	No. of Studies	No. of Controls	Overall			Serous Borderline Tumors			Mucinous Borderline Tumors		
			No. of Cases[a]	pOR[b]	95% CI	No. of Cases[a]	pOR[b]	95% CI	No. of Cases[a]	pOR[b]	95% CI
PID status	11										
Never had PID		12,755	2,153	1.00	Referent	1,184	1.00	Referent	891	1.00	Referent
Ever had PID		929	201	1.32	1.10, 1.58	114	1.43	1.14, 1.79	79	1.28	0.97, 1.68
Age at first PID episode, years	10										
Never had PID		11,679	1,976	1.00	Referent	1,101	1.00	Referent	804	1.00	Referent
<20		172	33	1.38	0.91, 2.09	16	1.28	0.73, 2.25	16	1.89	1.06, 3.35
20–29		355	87	1.52	1.17, 1.97	52	1.72	1.25, 2.38	32	1.60	0.94, 2.70
≥30		283	50	1.24	0.90, 1.73	27	1.38	0.89, 2.12	20	1.46	0.89, 2.40
P-trend					0.29			0.25			0.96
Per 1-year increment[c]				0.99	0.97, 1.01		0.98	0.96, 1.01		1.00	0.97, 1.03
Time since first PID episode, years	10										
Never had PID		11,679	1,976	1.00	Referent	1,101	1.00	Referent	804	1.00	Referent
<10		86	18	1.44	0.76, 2.73	12	1.74	0.86, 3.53	5	3.05	1.11, 8.40
10–19		159	48	1.73	1.21, 2.49	21	1.62	0.98, 2.70	25	2.37	1.46, 3.87
≥20		565	104	1.29	1.01, 1.64	62	1.48	1.09, 2.02	38	1.27	0.86, 1.86
P-trend					0.44			0.60			0.92
Per 5-year increment[c]				1.03	0.95, 1.12		1.03	0.89, 1.20		0.99	0.88, 1.12
No. of PID episodes	4										
0		3,287	662	1.00	Referent	349	1.00	Referent	282	1.00	Referent
1		142	25	0.88	0.55, 1.39	17	1.11	0.63, 1.95	8	0.84	0.33, 2.14
≥2		70	24	2.14	1.08, 4.24	12	3.28[d]	0.86, 12.54	11	1.98	0.80, 4.88

Abbreviations: CI, confidence interval; PID, pelvic inflammatory disease; pOR, pooled odds ratio.

[a] Numbers may not add up to totals due to missing values.

[b] Adjusted for parity (ever/never pregnant and number of pregnancies), oral contraceptive use (ever/never use and duration of use), and family history of ovarian or breast cancer (yes/no).

[c] Among women with a history of PID.

[d] *P* for heterogeneity < 0.05.

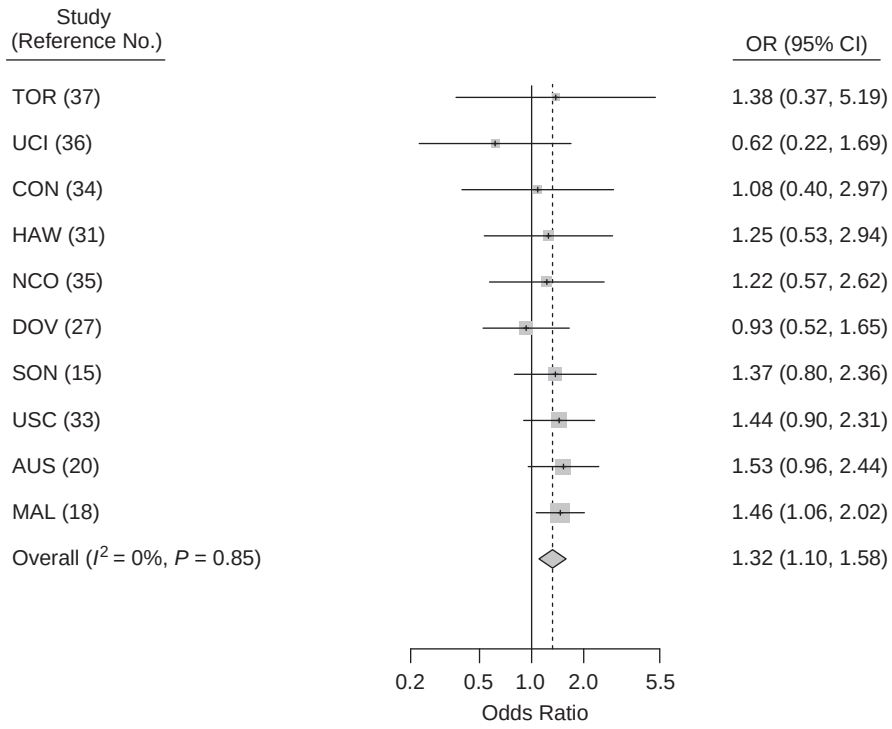

Figure 2. Associations between pelvic inflammatory disease (PID) status and the risk of borderline ovarian tumors among the pooled participants of 13 case-control studies in Australia, Europe, and North America, conducted between 1989 and 2009. Results are presented according to study site and overall and are adjusted for age, parity, oral contraceptive use (ever/never use and duration of use), and family history of ovarian or breast cancer (yes/no). For 2 of the studies (MAL and SON) results for the association between PID and the risk of borderline ovarian tumors have been published previously (15, 18). For the remaining studies, results for the association between PID and the risk of borderline ovarian tumors have not been published previously, and their references therefore refer to papers with general information about these studies (20, 27, 31, 33–37). For the present study, we obtained individual-level data from all studies directly through the Ovarian Cancer Association Consortium database. Each square and line represent the represent the odds ratio (OR) and 95% confidence interval (CI), respectively, and the size of the square indicates the study weighting. AUS, Australian Ovarian Cancer Study and Australian Cancer Study (Ovarian Cancer); CON, Connecticut Ovarian Cancer Study; DOV, Diseases of the Ovary and Their Evaluation; HAW, Hawaii Ovarian Cancer Study; HOP, Hormones and Ovarian Cancer Prediction; MAL, Danish Malignant Ovarian Tumor Study; NCO, North Carolina Ovarian Cancer Study; NJO, New Jersey Ovarian Cancer Study; NTH, Nijmegen Polygene Study and Nijmegen Biomedical Study; SON, Southern Ontario Ovarian Cancer Study; TOR, Familial Ovarian Tumor Study; UCI, University of California Irvine Ovarian Cancer Study; USC, Los Angeles County Case-Control Studies of Ovarian Cancer.

sites (MAL, USC, AUS, and SON) that were included in the present analysis (15, 18, 20, 23); results from those studies will not be discussed further. We found a 32% higher risk of borderline ovarian tumors associated with a history of PID, and risk estimates above unity were noted for nearly all individual studies. Furthermore, we observed similarly increased risks of serous and mucinous borderline tumors associated with PID status. Our novel finding of a 2-fold higher risk among women with multiple PID episodes may reflect a true association between PID and the risk of borderline ovarian tumors rather than being caused by chance or bias. Only 2 studies (SON and MAL, both included in the present analyses) have previously investigated the association between PID and the risk of borderline tumors (15, 18).

In the present study, the lack of any marked associations between PID and the risk of ovarian cancer overall is consistent with results from 1 case-control study (22), whereas 2 other studies found an increased risk of ovarian cancer (16, 17). Additionally, 2 studies assessed PID in relation to ovarian cancer risk but provided results only for ovarian cancer and borderline tumors combined, thereby hampering a comparison with the present results (19, 21); Ness et al. (21) reported null findings, and McAlpine et al. (19), in a Canadian cohort study, reported a 4-fold higher risk of ovarian cancer among women who had had PID. Concerning the histotypes of ovarian cancer, indications of an increased risk of low-grade serous cancer with PID were noted in the main analysis. Conversely, no convincing associations between PID and the risk of high-grade serous, mucinous, clear cell, or endometrioid ovarian cancer were noted in the main analyses. However, sensitivity analyses revealed statistically significantly increased risks of low-grade serous and endometrioid ovarian cancers when using data from the North American studies only. Other than 2 studies already included in the present pooled

analysis, no previous studies have assessed the association between PID and the risk of ovarian cancer according to histotype. Although we cannot completely rule out the possibility that these histotype-specific findings may be due to chance, the present study is the first, to our knowledge, to indicate differences in the risk profile of ovarian cancer histotypes with regard to PID. However, the low number of exposed cases for most of the histotypes limited the precision of the risk estimates, and our results must therefore be confirmed by others.

Nevertheless, our results suggest that PID may be differentially associated with the risk of ovarian tumors. Reasons for this difference are not known, but they may be associated with different pathogeneses of the ovarian tumor histotypes. Recently, the so-called dualistic model of ovarian carcinogenesis proposed that borderline tumors are precursors of type 1 (low-grade) ovarian cancers but unrelated to type 2 (high-grade) ovarian cancers (46). According to this hypothesis, type 1 tumors include low-grade serous and mucinous carcinomas, and these are believed to develop along a continuum of tumor progression from adenoma to borderline tumor to invasive carcinoma (46). Clear cell and low-grade endometrioid carcinomas are also type 1 cancers and are believed to develop from endometriosis. Our results demonstrated an association between PID and the risk of borderline ovarian tumors and indicated that the risk of low-grade serous cancer might also be increased, which accords well with the theory of a stepwise development from a serous borderline tumor to low-grade serous cancer. In contrast, no associations between PID and high-grade serous ovarian cancer were observed. Therefore, our results suggest that PID is a risk factor for borderline and possibly also low-grade serous ovarian cancer, whereas no marked associations were observed for the other histotypes of ovarian cancer. The possible underlying biological mechanisms responsible for this differential association between PID and ovarian tumor types are unknown and require further investigation in epidemiologic and biological studies.

A strength of the present study is the use of pooled data from 13 case-control studies. The large sample size resulted in increased statistical power and enabled us to estimate risks according to invasiveness and histotype. Moreover, all the studies we included were population-based, and information on PID was obtained through in-person interviews in the majority of them. In addition, we used individual-level data carefully harmonized and entered into a single data set. The use of a 2-stage approach (39) enabled us to account for differences in design and data collection between studies and to control for several potential confounders. Finally, all studies with the relevant exposure data in OCAC were included regardless of their individual results, thus removing the influence of publication bias.

Some limitations should also be mentioned. First, information about PID status was self-reported in all studies, and the proportion of control participants reporting an episode of PID in the individual studies ranged from 0.4% to 27%. Unfortunately, most studies had no data or insufficient data on treatment for PID, which could have added important information in terms of validating the PID diagnoses. The highest frequencies were reported in the Danish study (MAL: 27%) and in a Canadian study (SON: 20%); the remaining 11 studies all had PID proportions below 6%. Reasons for the differences in proportions among the studies may include geographic variation in the prevalence of PID-causing pathogens, different phrasing of the PID-related questions, or differences in the prevalence of high-risk sexual behaviors. However, we believe that underestimation of PID exposure is the most likely cause for the low proportions of women with a history of PID in the majority of studies, because previous studies from Sweden and the United States have estimated lifetime prevalences of PID between 6% and 20% (12 14). In studies with self-reported data on PID exposure, including the present study, the true proportion of women who have had PID might be underestimated for several reasons women might have forgotten about a past PID episode, chosen not to report it, or had unrecognized, subclinical PID. Hence, we cannot rule out the possibility that this misclassification of PID status could have influenced our results. Interestingly, investigators in only 2 previous studies did not use self-reported data on PID but instead obtained information on PID from a population-based health insurance database or used evidence of inflammation at surgery for tubal damage as a proxy for previous PID, and both groups reported an increased risk of ovarian cancer associated with PID (17, 19). Therefore, in future studies, researchers should consider using a more objective measure of PID, such as data obtained from reliable health registries or through serological testing for antibodies to PID-causing pathogens, including *Chlamydia trachomatis* and *Neisseria gonorrhoeae*.

Second, misclassification of PID exposure might also result when women mistakenly report bladder or vaginal infections as PID. However, we expect this misclassification to be relatively infrequent, because in the majority of included studies, PID was defined as diagnosed by a physician, or the question specified that bladder or vaginal infections were not included. Furthermore, the majority of studies performed in-person interviews, thus allowing for potential uncertainties to be clarified. Third, the retrospective design of case-control studies introduces the potential for recall bias, in which case patients are more likely than control participants to report past exposures. However, we would not expect such overreporting to be differential with respect to degree of invasiveness of diagnosed ovarian tumors, and we therefore do not believe that this can explain the increased risk we observed for borderline tumors but not for ovarian cancer. Fourth, surveillance bias is potentially of concern, because women with PID symptoms may undergo ultrasonography or laparoscopy during which the ovaries are visualized, leading to coincidental findings of ovarian tumors. However, this potential surveillance bias is probably minimal, because our sensitivity analyses excluding women with PID less than 1 3 years in the past revealed virtually identical results as in the main analyses. Fifth, only 5 studies had information on the number of PID episodes, and the absence of thorough information on this exposure variable limited our ability to fully investigate and interpret any potential dose-response associations between number of PID episodes and the risk of ovarian cancer and borderline ovarian tumors. Finally, despite the

large study size, we still had limited statistical power because of small proportions of women with PID in some of the categorical analyses and for some of the rarer histotypes, and we cannot completely rule out the possibility that some of the observed associations may have been be due to the large number of comparisons; thus our results should be interpreted with caution.

In conclusion, in this large, pooled analysis, we observed an increased risk of borderline ovarian tumors among women with a history of PID. These risks increased with the number of PID episodes. Conversely, we found no association between PID and the risk of ovarian cancer overall, but indications of an increased risk of low-grade serous cancer were noted. These findings suggest that PID may be a risk factor for borderline ovarian tumors and possibly for low-grade serous cancer, although no convincing associations were seen for other ovarian cancer histotypes. However, until the specificity of the association is confirmed in additional epidemiologic and biological studies, the association between PID and ovarian cancer risk is still somewhat uncertain.

ACKNOWLEDGMENTS

Author affiliations: Virus, Lifestyle and Genes Unit, Danish Cancer Society Research Center, Copenhagen, Denmark (Christina B. Rasmussen, Susanne K. Kjaer, Estrid Høgdall, Allan Jensen); Department of Gynecology, Rigshospitalet, University of Copenhagen, Copenhagen, Denmark (Susanne K. Kjaer); Statistics, Bioinformatics and Registry Unit, Danish Cancer Society Research Center, Copenhagen, Denmark (Vanna Albieri); Cancer Prevention and Control Program, Rutgers Cancer Institute of New Jersey, Rutgers, State University of New Jersey, New Brunswick, New Jersey (Elisa V. Bandera); Department of Epidemiology, Geisel School of Medicine, Dartmouth College, Lebanon, New Hampshire (Jennifer A. Doherty); Department of Community and Family Medicine, Geisel School of Medicine, Dartmouth College, Lebanon, New Hampshire (Jennifer A. Doherty); Molecular Unit, Department of Pathology, Herlev Hospital, University of Copenhagen, Copenhagen, Denmark (Estrid Høgdall); Population Health Department, QIMR Berghofer Medical Research Institute, Herston, Queensland, Australia (Penelope M. Webb, Susan J. Jordan); Program in Epidemiology, Division of Public Health Sciences, Fred Hutchinson Cancer Research Center, Seattle, Washington (Mary Anne Rossing, Kristine G. Wicklund); Department of Epidemiology, School of Public Health, University of Washington, Seattle, Washington (Mary Anne Rossing); Cancer Prevention and Control, Samuel Oschin Comprehensive Cancer Institute, Cedars-Sinai Medical Center, Los Angeles, California (Marc T. Goodman); Community and Population Health Research Institute, Department of Biomedical Sciences, Cedars-Sinai Medical Center, Los Angeles, California (Marc T. Goodman); Division of Gynecologic Oncology, Department of Obstetrics, Gynecology and Reproductive Sciences, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania (Francesmary Modugno, Robert P. Edwards); Ovarian Cancer Center of Excellence, Magee-Women's Research Institute and Foundation, Pittsburgh, Pennsylvania (Francesmary Modugno, Robert P. Edwards); Women's Cancer Research Center, University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania (Francesmary Modugno, Robert P. Edwards); Department of Epidemiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania (Francesmary Modugno); Department of Cancer Prevention and Control, Roswell Park Cancer Institute, Buffalo, New York (Kirsten B. Moysich); School of Public Health, University of Texas, Houston, Texas (Roberta B. Ness); Department of Public Health Sciences, School of Medicine, University of Virginia, Charlottesville, Virginia (Joellen M. Schildkraut); Department of Obstetrics and Gynecology, Duke University Medical Center, Durham, North Carolina (Andrew Berchuck); Department of Epidemiology and Biostatistics, Memorial Sloan Kettering Cancer Center, New York, New York (Sara H. Olson); Department for Health Evidence, Radboud University Medical Center, Nijmegen, the Netherlands (Lambertus A. Kiemeney); Department of Urology, Radboud University Medical Center, Nijmegen, the Netherlands (Lambertus A. Kiemeney); Department of Obstetrics and Gynaecology, Radboud University Medical Center, Nijmegen, the Netherlands (Leon F. A. G. Massuger); Women's College Research Institute, University of Toronto, Toronto, Ontario, Canada (Steven A. Narod); Department of Cancer Epidemiology, Moffitt Cancer Center, Tampa, Florida (Catherine M. Phelan); Department of Epidemiology, Genetic Epidemiology Research Institute, Center for Cancer Genetics Research and Prevention, School of Medicine, University of California, Irvine, Irvine, California (Hoda Anton-Culver); Department of Epidemiology, School of Medicine, University of California, Irvine, Irvine, California (Argyrios Ziogas); Department of Preventive Medicine, Norris Comprehensive Cancer Center, Keck School of Medicine, University of Southern California, Los Angeles, California (Anna H. Wu, Celeste L. Pearce); Department of Epidemiology, School of Public Health, University of Michigan, Ann Arbor, Michigan (Celeste L. Pearce); and Department of Chronic Disease Epidemiology, Yale School of Public Health, Yale University, New Haven, Connecticut (Harvey A. Risch).

This work was supported by the European Commission's Seventh Framework Programme grant 223175 (HEALTH-F2-2009-223175). It was also supported by the US National Institutes of Health (grants R01 CA074850 and R01 CA080742 (CON); R01 CA112523 and R01 CA87538 (DOV); R01 CA58598, N01 CN55424, and N01 PC 67001 (HAW); R01 CA95023, K07 CA080668, and P50 CA159981 (HOP); R01 CA61107 (MAL); R01 CA76016 (NCO); P30 CA072720, K07 CA095666, R01 CA83918, and K22 CA138563 (NJO); R01 CA063678, R01 CA149429, and R01 CA063682 (TOR); R01 CA058860, R01 CA092044, and PSA 042205 (UCI); P01 CA17054, P30 CA14089, R01 CA61132, N01 PC67010, R03 CA113148, R03

CA115195, and N01 CN025403 (USC)); Danish Cancer Society (grant 94 222 52 (MAL)); Mermaid 1 (MAL); US Army Medical Research and Materiel Command (grant DAMD17-01-1-0729) (AUS); National Health and Medical Research Council of Australia (grants 199600 and 400281 (AUS)); Cancer Councils of New South Wales, Victoria, Queensland, South Australia, and Tasmania (AUS); Cancer Foundation of Western Australia (AUS); US Department of Defense (grants DAMD17-02-1-0669 (HOP) and DAMD17-02-1-0666 (NCO)); Cancer Institute of New Jersey (NJO); Radboud University Nijmegen Medical Centre (NTH); Lon V Smith Foundation (grant LVS-39420 (UCI)); and California Cancer Research Program (grants 00-01389V-20170 and 2II0200 (USC)). The Ovarian Cancer Association Consortium is supported by a grant from the Ovarian Cancer Research Fund.

Penelope Webb and Susan Jordan wrote on behalf of the Australian Ovarian Cancer Study Group. The Australian group thanks all the clinical and scientific collaborators for their contribution (AUS). The cooperation of the 32 Connecticut hospitals, including Stamford Hospital, in allowing patient access is gratefully acknowledged (CON). The MAL study is grateful to Nick Martinussen for data management assistance. We are grateful to the family and friends of Kathryn Sladek Smith for their generous support of the Ovarian Cancer Association Consortium through their donations to the Ovarian Cancer Research Fund.

Certain data from the Connecticut Ovarian Cancer Study were obtained from the Connecticut Tumor Registry, Connecticut Department of Public Health. The Connecticut Ovarian Cancer Study investigators assume full responsibility for analyses and interpretation of these data.

Conflict of interest: none declared.

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Exhibit 110

Cancer Epidemiology 55 (2018) 110–116

Contents lists available at ScienceDirect

Cancer Epidemiology

journal homepage: www.elsevier.com/locate/canep

Risk of high-grade serous ovarian cancer associated with pelvic inflammatory disease, parity and breast cancer

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ARTICLE INFO

Keywords:
Pelvic inflammatory disease
Parity
Endometriosis
Infertility
Hysterectomy
Tubal ligation
Ovarian neoplasms
Breast neoplasms
Cohort studies
Risk

ABSTRACT

Background: Ovarian carcinoma is not a single disease, but rather a collection of subtypes with differing molecular properties and risk profiles. The most common of these, and the subject of this work, is high-grade serous ovarian carcinoma (HGSC).

Methods: In this population-based study we identified a cohort of 441,382 women resident in Western Australia who had ever been admitted to hospital in the State. Of these, 454 were diagnosed with HGSC. We used Cox regression to derive hazard ratios (HRs) comparing the risk of disease in women who had each of a range of medical diagnoses and surgical procedures with women who did not.

Results: We found an increased risk of HGSC associated with a diagnosis of pelvic inflammatory disease (PID) (HR 1.47, 95% CI 1.04–2.07) but not with a diagnosis of infertility or endometriosis with HRs of 1.12 (95% CI 0.73–1.71) and 0.82 (95% CI 0.55–1.22) respectively. A personal history of breast cancer was associated with a three-fold increase in the rate of HGSC. Increased parity was associated with a reduced risk of HGSC in women without a personal history of breast cancer (HR 0.57; 95% CI 0.44-0.73), but not in women with a personal history of breast cancer (HR 1.48; 95% CI 0.74–2.95).

Conclusions: Our finding of an increased risk of HGSC associated with PID lends support to the hypothesis that inflammatory processes may be involved in the etiology of HGSC.

1. Introduction

Epithelial carcinomas account for 90% of all ovarian cancers and have been classified into five major histological subtypes: high grade serous, low grade serous, endometrioid, clear cell and mucinous. These subtypes are different diseases with differing molecular, histopathological and clinical characteristics [1–3] and risk factors [4]. For this reason, it is important that associations between risk factors and disease are established separately for each subtype. Of all the histological subtypes, high grade serous tumours (including ovarian, fallopian tube and primary peritoneal carcinomas) (HGSC) are the most common, representing around 70% of all carcinomas [3].

Numerous studies have evaluated the association between established risk factors and ovarian cancer overall (reviewed in [5–7]). Many have also examined these associations separately for each subtype [8–31]. Those that have done so have generally grouped high and low grade serous subtypes together into a single category: serous ovarian cancer. It is now recognised that high and low grade serous carcinoma are two different tumour types [2]. Few studies have examined the associations with HGSC. Among six such studies, two identified

Abbreviations: BO, bilateral oophorectomy; BSO, bilateral salpingo-oophorectomy; CI, confidence interval; HGSC, high grade serous ovarian carcinoma; HR, hazard ratio; ICD, International Classification of Diseases; PID, pelvicinflammatory disease; USO, unilateral salpingectomy, oophorectomy or salpingo-oophorectomy; WA, Western Australia

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https://doi.org/10.1016/j.canep.2018.05.011
Received 2 March 2018; Received in revised form 18 May 2018; Accepted 27 May 2018

ovarian, fallopian tube and peritoneal cancers and classified tumours according to histological pathways; one [11] grouped together invasive ovarian, fallopian tube and peritoneal serous cancers with high grade endometrioid and undifferentiated tumours into the Type 2 category [11,19], whilst another [19] created three categories and grouped high grade serous together with undifferentiated tumours. A third classified serous ovarian and peritoneal cancers into three grades: well, moder ately and poorly differentiated [30]. Three others considered high grade serous tumours of the ovary but did not mention fallopian tube or peritoneal tumours [12,13,22].

It is generally established that increasing parity is associated with a reduced risk of ovarian cancer, although this association differs across subtypes with the greatest reduction in risk for endometrioid and clear cell, the least reduction for serous and some heterogeneity across stu dies of mucinous subtypes [8,9,11,12,14,16,17,19,21,26,28 31]. It is possible that the different hormonal milieu seen in women carrying multiple pregnancies may lead to a modification in ovarian cancer risk, although most studies have not found an association between ovarian cancer overall and twin pregnancies [32 39].

Endometriosis appears to be associated with an increased risk of endometrioid and clear cell subtypes [19,20]; possibly associated with an increased risk of low grade serous but perhaps not high grade serous tumours [22,30].

Findings with regard to tubal ligation are contradictory: some find a reduced risk of serous ovarian cancer [10,16,21,24,26] whilst others do not [14,18,25,27,30]. An early study by Risch [26] examining the as sociation between hysterectomy and serous ovarian cancer found a reduced risk, though later studies have generally not found an asso ciation [11,14,19,21,25,30].

It has been speculated that chronic inflammation resulting from pelvic inflammatory disease (PID) may play a role in ovarian carcino genesis [40]. This association has been investigated by a number of authors, with contradictory findings. Risch et al. [26] found that self reported recurrent PID was associated with an increased risk of ovarian cancer overall, whereas Ness et al. [41] found only a weak association between the two. A pooled analysis of individual level data did not find an association between self reported PID and either serous or high grade serous ovarian cancer [42]. A subsequent record linkage study [23] found an association between hospital diagnosed PID and serous ovarian cancer.

A family history of breast or ovarian cancer, usually in the mother or sister, has often been included in multivariable analyses of risk fac tors for serous [9,16,21,30], and high grade serous [30] ovarian cancer. Identifying cancers in only first degree relatives may underestimate risk as it does not take into account inheritance of cancer susceptibility genes from the paternal line. A personal history of breast cancer has generally not been investigated.

The aim of the present study was to examine the association be tween HGSC and a number of ovarian cancer risk factors, including parity, plurality (the delivery of twins and higher order multiples), endometriosis, infertility, PID, hysterectomy, unilateral salpingo oo phorectomy, tubal ligation and a personal history of breast cancer.

2. Methods

2.1. The study population

This study was conducted in Western Australia (WA), the largest state in Australia with a geographic area of 2,529,875 square kilometres and a population of 2.59 million (11% of the total Australian popula tion). The majority of the population resides in the south west corner of the state.

This was a population based cohort study. The study population included all women, born between 1945 and 1975 inclusive, residing in WA, who had been admitted to hospital in WA at any time between 1 January 1980 and 30 June 2014. Hospital records for these women extended back to 1 January 1970. We used WA's Hospital Morbidity Data Collection [43] to identify the study population and also to define many of the exposure variables. The remaining exposure variables and the outcome variable were identified through linkage to other state wide demographic and health databases using WA's data linkage system [44]. Linkage to the WA Deaths Register enabled the identification of deaths to allow for censoring in survival analysis. Linkage to the WA Midwives Notification System allowed the identification of births and parity related variables from 1980; the WA Births Register was used to identify births in the period 1970 1980. The WA Electoral Roll, with information available from 1988 onward, was used to identify women who were not registered to vote or who had moved interstate. Ovarian cancer and breast cancer cases were identified from the WA Cancer Registry.

The state of WA, although geographically isolated, has a dynamic population, experiencing both inward and outward migration. This has the potential to lead to bias due to misclassification of early exposures in women who migrate into the state, and loss to follow up in women who migrate out. For this reason, we excluded women whose hospital records showed they were overseas visitors or resided out of the state, and women whose cancer records showed that ovarian cancer was di agnosed out of the state. We also excluded women for whom we did not have WA Electoral Roll records, and women whose Electoral Roll re cords showed they had moved out of the state (Fig. 1). With only a few exceptions, all Australian citizens are required to register on the Elec toral Roll and to update their residential address soon after moving [45]. We also conducted comparative and sensitivity analyses to assess the impact of inward and outward migration on our risk estimates, comparing the final cohort of women which included only those known to be resident in WA with a larger preliminary cohort that also included women who were not known to be WA residents.

2.2. Exposure variables

We examined the association between HGSC and diagnoses of in fertility, endometriosis and PID; parity and plurality (the delivery of twins and higher order multiples); tubal ligation; hysterectomy without salpingectomy or oophorectomy; unilateral salpingectomy, oophor ectomy or salpingo oophorectomy (USO) without hysterectomy and a personal history of breast cancer. We did not examine the association with hysterectomy plus USO.

Diagnoses and procedures were recorded in the Hospital Morbidity Data Collection and coded according to contemporaneous International Classification of Diseases (ICD) codes, including ICD 8, ICD 9 and ICD 10 AM diagnostic codes and COSO (Code of Surgical Operations), ICPM (International Classification of Procedures in Medicine), ICD 9 and ACHI (Australian Classification of Health Interventions) procedure codes. Diagnostic and procedure codes used to identify each study variable are listed in Supplementary Table 1. We included any mention of the diagnosis or procedure whether it was recorded as a principal or additional diagnosis or a principal or additional procedure. Exposure variables were reported as categorical time varying binary variables, with exposure changing from 0 to 1 (unexposed to exposed) at the date of the relevant diagnosis or procedure or birth of third child. We compared women whose hospital records mentioned the diagnosis or procedure, with women whose hospital records had no mention of the diagnosis or procedure.

We used a binary classification for parity, comparing women who delivered 3 or more children with women who delivered 0, 1 or 2. The reason for this was because of the possibility of misclassification of parity in women who only gave birth before 1970 or out of WA. Women who gave birth either prior to 1970 or out of WA and did not have any subsequent deliveries in WA were classified as nulliparous because we did not have any information on deliveries in these women. Women who gave birth out of WA or prior to 1970 and had subsequent de liveries in WA were correctly classified. We reasoned that this

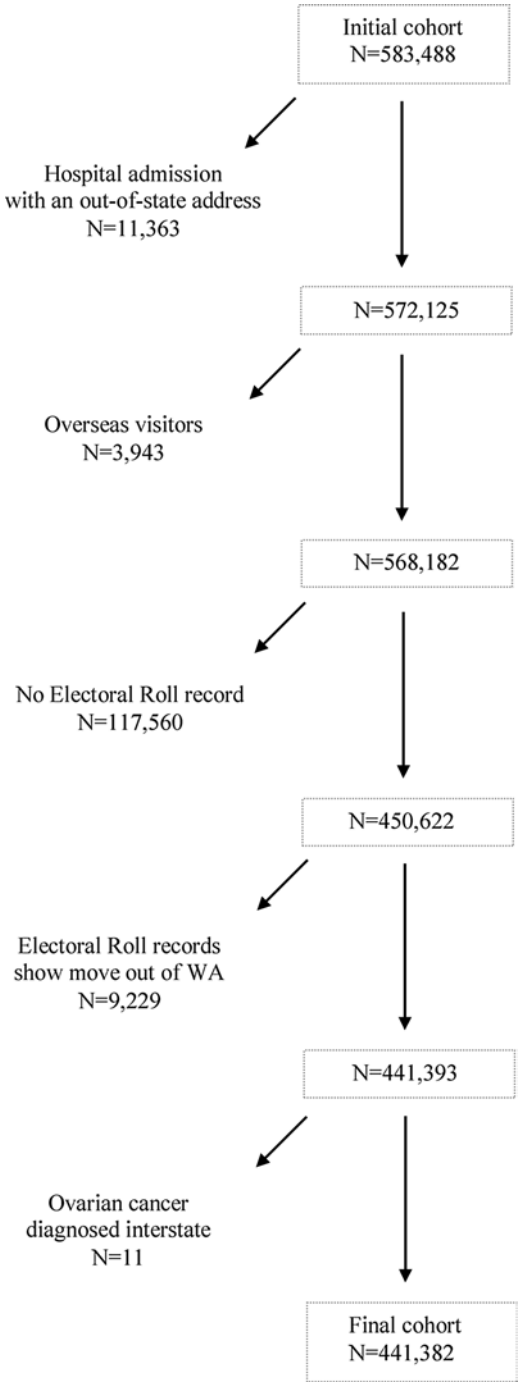

Fig. 1. Flow chart showing cohort selection with eligible and ineligible study participants.

misclassification was much less likely in women who delivered three or more children than in women who delivered one or two. Preliminary investigation and sensitivity analyses supported this assumption.

Plurality was included as a binary variable, with women who delivered twins, triplets and higher order multiples grouped together.

We also considered a personal history of breast cancer. Cases were identified from the WA Cancer Registry (from 1982), with earlier cases (between 1970 and 1982) identified from the Hospital Morbidity Data Collection.

Table 1
Characteristics of the study population.

Characteristic	
Number of women	441,382
Total duration of follow-up (person-years)	23,206,070
Mean age at end of follow-up (years), (SD)	52.6 (8.7)
Number of women diagnosed with HGSC	454
Median year of HGSC diagnosis, (range)	2008 (1987-2014)
Mean age at diagnosis of HGSC (years) (SD)	54.4 (7.5)
Infertility	
Number (%) with a diagnosis of infertility	28,859 (6.5%)
Mean age at first infertility diagnosis (years), (SD)	32.7 (5.7)
Number with a diagnosis of infertility and a later diagnosis of HGSC	24
Endometriosis	
Number (%) with a diagnosis of endometriosis	34,552 (7.8%)
Mean age at first endometriosis diagnosis (years), (SD)	38.4 (8.2)
Number with a diagnosis of endometriosis and a later diagnosis of HGSC	28
PID	
Number (%) with a diagnosis of PID	33,335 (7.6%)
Mean age at first PID diagnosis (years), (SD)	34.4 (9.4)
Number with a diagnosis of PID and a later diagnosis of HGSC	39
Hysterectomy without salpingectomy or oophorectomy	
Number who had a hysterectomy (%)	47,947 (10.9%)
Mean age at hysterectomy (years), (SD)	41.5 (7.4)
Number with a hysterectomy and a later diagnosis of HGSC	64
USO, US or UO without hysterectomy	
Number who had a USO (%)	6887 (1.6%)
Mean age at USO (years), (SD)	39.5 (10.2)
Number with a USO and later diagnosis of HGSC	< 10[1]
Tubal ligation	
Number who had tubal ligation (%)	78,639 (17.8%)
Mean age at tubal ligation (years), (SD)	33.7 (5.4)
Number with tubal ligation and a later diagnosis of HGSC	85
Plural delivery	
Number who had a plural delivery	9,490 (2.2%)
Mean age at first plural delivery (years), (SD)	30.1 (5.6)
Number with a plural delivery and a later diagnosis of HGSC	12
Parity	
Number (%) with three or more births	123,777 (28.0%)
Mean age at birth of third child (years), (SD)	30.4 (5.0)
Number who had three or more births and were later diagnosed with HGSC	87
Personal history of breast cancer	
Number (%) with a personal history of breast cancer	12,168 (2.8%)
Mean age at breast cancer diagnosis (years), (SD)	49.6 (8.5)
Number with a personal history of breast cancer and a later diagnosis of HGSC	37

[1] Actual number not presented in accordance with data confidentiality agreement.

2.3. Outcome variable

The outcome of interest was a diagnosis of HGSC. Data were obtained from the WA Cancer registry and classification of ovarian cancer subtypes was reviewed and revised where appropriate [46]. Consequently, HGSC included correctly classified ovarian, tubal and peritoneal high grade serous carcinomas.

2.4. Data analysis

Data were analysed using Cox regression with time varying covariates. We used age as the time scale. Women were followed from birth until the censor date of 30 June 2014, or a diagnosis of HGSC or any type of ovarian cancer, or death, whichever came first. Follow up was also censored at the time of bilateral salpingo oophorectomy (BSO) or

Table 2
Association between risk factors and the rate of HGSC. Unadjusted and adjusted hazard ratios (HRs) with 95% confidence intervals (CIs).

Exposure[1]	Unadjusted HR[2] (95% CI)	Adjusted HR[3] (95% CI)
Infertility	1.24 (0.82-1.88)	1.12 (0.73-1.71)
Endometriosis	0.85 (0.58-1.25)	0.82 (0.55-1.22)
PID	1.36 (0.98-1.89)	1.47 (1.04-2.07)
Hysterectomy [4]	0.89 (0.68-1.16)	0.94 (0.71-1.24)
USO[5]	0.55 (0.20-1.46)	0.48 (0.18-1.29)
Tubal ligation	0.78 (0.62-0.99)	0.83 (0.65-1.06)
Plural delivery[6]	1.48 (0.84-2.63)	1.59 (0.89-2.82)
High parity	0.61 (0.48-0.77)	
Breast cancer	3.54 (2.52-4.98)	
Parity and breast cancer[7]		
No breast cancer, low parity[8]		1.00 (ref)
No breast cancer, high parity[9]		0.57 (0.44-0.73)
Breast cancer, low parity		2.79 (1.85-4.20)
Breast cancer, high parity		4.14 (2.32-7.39)

[1] Reference levels for each variable are for the unexposed. For example, for infertility, the HR for the group of women who had no mention of infertility in any hospital record was 1.00.

[2] Age is used as the time scale, hence unadjusted HRs are effectively age-adjusted.

[3] Each HR is derived from a model that includes all the listed variables.

[4] Hysterectomy without oophorectomy or salpingectomy.

[5] Unilateral salpingectomy, oophorectomy or salpingo-oophorectomy without hysterectomy.

[6] The delivery of twins and higher order multiples.

[7] There was significant interaction between parity and breast cancer ($p = 0.015$). Therefore, results for parity and breast cancer are presented separately at each level of each variable.

[8] Women of low parity have had 0, 1 or 2 births.

[9] Women of high parity have had 3 or more births.

bilateral oophorectomy (BO); unilateral oophorectomy after a previous unilateral oophorectomy; hysterectomy with BSO or BO, or hysterectomy where salpingo oophorectomy was not specified as bilateral or unilateral. All exposure variables were time varying, that is, the value of the binary exposure variables changed from 0 to 1 at the time of exposure.

Data were analyzed first in unadjusted models and then in multivariable models. Because age was used as the time scale, all models were effectively age adjusted. All variables included in the multivariable model are listed in Table 2. We checked for interactions between all the study variables. We tested the proportional hazards assumption by examining Schoenfeld residuals in the initial univariate and final multivariable models and did not find any evidence for an overall violation of the assumption.

Because age was used as the time scale, hazard rates could be interpreted as age specific incidence rates of HGSC. However, the semi-parametric Cox model produces only relative hazard rates (hazard rate ratios) rather than hazard rates. To visualise age specific incidence rates of HGSC by parity in this cohort, flexible parametric proportional hazards (Royston Parmar) models were employed [47]. Spline functions with one internal knot were used to model the baseline hazards, while parity was included as a time dependent spline function with one internal knot. This allowed us to investigate subtle changes in the proportionality of hazards with age which we have represented graphically.

Data were analysed using SPSS version 24 (IBM) and Stata version 14 (StataCorp, College Station, Texas).

2.5. Ethics

This study was approved by Department of Health Custodians, the WA Department of Health Ethics Committee and Curtin University and The University of Notre Dame Human Research Ethics Committees.

3. Results

3.1. The cohort

A total of 583,488 women, born between 1 January 1945 and 31 December 1975 had a hospital admission in WA between 1 January 1980 and 30 June 2014. We excluded women not known to be resident in WA, as described in Fig. 1, and derived a final cohort numbering 441,382.

A total of 454 women were diagnosed with HGSC. The average age at diagnosis was 54.4 years (Table 1); (median 55.4 years).

Endometriosis was the most common gynecological diagnosis, identified in 7.8% of women in our cohort. PID was diagnosed in 7.6% of women and infertility in 6.5%. The most common gynaecological surgery was tubal ligation, in 17.8% of the women. Breast cancer was identified in 2.8% of women (Table 1).

3.2. Association between risk factors and HGSC

We first examined the association between exposure variables and the rate of HGSC in unadjusted analysis, and then included all exposure variables in the final multivariable model (see Table 2).

Neither infertility nor endometriosis appeared to be associated with an increased risk of HGSC in either unadjusted or adjusted analyses. Women in our cohort diagnosed with PID had a 47% increased rate of HGSC compared to women with no such diagnosis (Table 2).

There did not appear to be an association between hysterectomy and HGSC. Tubal ligation was associated with a 17% reduction in risk but confidence intervals included one (Table 2).

The delivery of twins and higher order multiples was associated with a 59% increased rate of HGSC with confidence intervals that also included one (Table 2).

Increased parity (3 or more births, compared with 0, 1 or 2 births) was associated with a reduced risk of HGSC whereas a personal history of breast cancer was associated with an increased risk. A total of 12,168 women had a personal history of breast cancer; of these, 37 had a later diagnosis of HGSC (Table 1). We observed a significant interaction between parity and breast cancer, and for this reason, present results for each category separately.

Among women without breast cancer, higher parity was associated with a 43% reduced rate of HGSC (Table 2). This was not the case in women with a history of breast cancer where the HR associated with higher parity was 1.48, (95% CI 0.74 2.95) (data not shown, but this can be estimated by dividing 4.14 by 2.79 (see Table 2)).

A personal history of breast cancer was associated with an almost 3 fold increase in the rate of HGSC in women of low parity (women with 0, 1 or 2 births) and a 4 fold increase in the rate of HGSC in women of high parity (women with 3 or more births) (Table 2).

Among women of high parity, a personal history of breast cancer was associated with a 7.30 times increased rate of HGSC (95% CI 3.96 13.46) (this can be estimated by dividing 4.14 by 0.57 (see Table 2)).

Spline functions were used to graph the association between a woman's age and the rate of HGSC. We compared women of low and high parity (women with 0, 1 or 2 births compared with women with 3 or more births). Age specific rates of HGSC were lower in women of higher parity throughout their middle years. The difference between parity groups diminished as women approached their mid 60 s (Fig. 2).

3.3. Comparative and sensitivity analyses

To estimate the impact of potential misclassification due to inward and outward migration, we conducted comparative and sensitivity analyses comparing findings from a cohort which included both WA

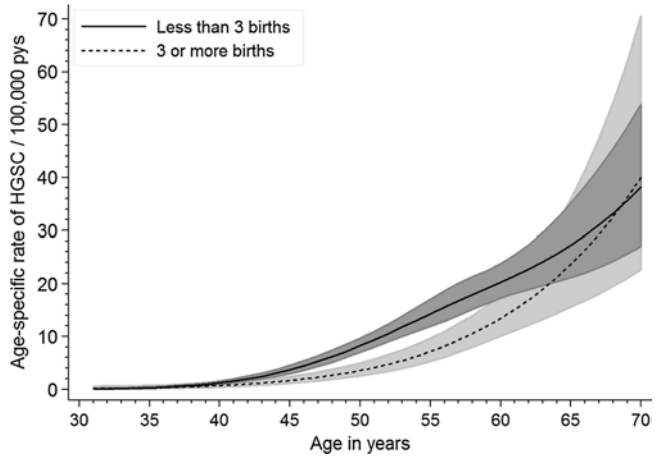

Fig. 2. Predicted age-specific rates of HGSC in women without a history of breast cancer stratified by periods of time having had less than three births and periods of time having had three or more births. Predictions adjusted for infertility, endometriosis, PID, hysterectomy, USO, sterilisation, plurality, parity and breast cancer and the interaction between parity and breast cancer. All the above variables set at 0 for the predicted parity rates. Shading represents 95%CI.

residents and non residents (n = 583,488) with our final cohort which included only women known to be WA residents (n = 441,382). Our overall conclusions were the same irrespective of which cohort we chose and HR estimates for all variables were similar. The estimate for higher parity (comparing women with 3 or more births with women with 0, 1 or 2 births) was slightly closer to the null in the larger cohort, which included women known to have migrated into or out of the state (the HR estimate was 0.67 in the preliminary cohort (95% CI 0.52 0.85), compared with 0.57 in the final cohort (95% CI 0.44 0.73)) suggesting there may have been some misclassification of parity in the larger cohort which we were able to reduce by restricting the cohort to known WA residents.

We also compared the results in Fig. 2 with those from a restricted cohort which excluded, firstly, nulliparous women, secondly, women born prior to 1950 and thirdly, women born prior to 1955. Our conclusions remained the same, suggesting that the findings could not be explained by parity misclassification.

4. Discussion

In this study we examined the association between high grade serous tubo ovarian carcinoma and a range of risk factors.

One of the factors we considered was a personal history of breast cancer. Breast cancer is a relatively common cancer [48], usually sporadic in nature [49]. In our study, the rate of HGSC in women with a personal history of breast cancer was around three times that of women without a breast cancer diagnosis. It is likely that at least some of these affected women carried a *BRCA* mutation, which is associated with an increased risk of both breast and ovarian cancer, particularly HGSC. There is currently no reliable screening test for the early detection of ovarian cancer [50,51]. As the cost of genetic testing becomes more affordable, it may be worthwhile expanding the criteria for women in whom genetic testing is appropriate. It may become cost effective to offer this simple saliva or blood test to more women treated for breast cancer.

We also examined the association between parity and risk of HGSC and found a reduced risk of HGSC in women of higher parity (i.e. women with 3 or more births, compared with women with 0, 1 or 2 births), consistent with most studies of serous [8,9,11,12,16,17,19,21,26,28 31] and high grade serous [19,30] ovarian carcinoma, but not all. Some studies did not find an association with serous [14,28] or high grade serous [14] carcinoma. Our analysis of age specific rates suggested that this parity associated risk reduction attenuates over time, so that as women approach their mid 60 s, the reduced risk associated with higher parity diminishes. This observation finds support from studies of McGuire et al. [52] and might help to explain why some studies have found that older age at delivery is associated with a reduced ovarian cancer risk [53,54] with a later delivery extending parity related protection further into middle age.

Among women with a personal history of breast cancer, there did not appear to be a reduced risk of HGSC associated with high parity. Instead, we found a 48% increase in the risk of HGSC in women of increased parity, although the statistical evidence was weak. Many studies have examined ovarian cancer risk in *BRCA* mutation carriers [55 57]. Some of these found a reduced risk of ovarian cancer associated with increasing parity in *BRCA1* carriers, but not in *BRCA2* carriers [55,56]. It is possible that the long held association between parity and ovarian cancer and in particular, HGSC, may not hold for all subgroups of the population, in particular, for older women and women with a personal history of breast cancer.

We did not find any evidence for an association between HGSC and hysterectomy without salpingo oophorectomy. This is consistent with most studies examining the association with serous ovarian cancer [11,14,21,25,30]. Few papers report the association specifically with HGSC, but where they do, they generally find a weaker association between hysterectomy and HGSC than between hysterectomy and other ovarian cancer subtypes [30].

We found a small, 17% reduction in risk of HGSC associated with tubal ligation, though with confidence intervals that included one. Findings from other groups are mixed; for example Gaitskell et al. [13] found a significant 23% reduction in risk of HGSC associated with tubal ligation whereas Wentzensen [30] found a non significant 9% reduction in the risk of serous ovarian cancer associated with tubal ligation. With regard to high grade serous ovarian cancer, Gaitskell et al. [13] found a reduced risk associated with tubal ligation whereas Merritt et al. [19] did not.

We found a reduced risk of HGSC associated with USO without hysterectomy, although this finding was based on only a few HGSC cases in the exposed. This was consistent with results from Rice et al. [25] but contrasts with our earlier findings of the relationship with ovarian cancer overall in a cohort of women undergoing investigation and treatment for infertility [58].

We did not detect an association between infertility and HGSC. In other studies, Merritt et al. [19] did not find an association between infertility and high grade serous carcinomas whereas Jensen et al. [15], but not Tung et al. [28] found an association between infertility and serous carcinomas. Our findings with regard to the relationship between HGSC and endometriosis are consistent with others [19,22,30].

We found that a diagnosis of PID was associated with an increased risk of HGSC. This finding was consistent with a recent record linkage cohort study by Rassmussen et al. [23] which found an increased risk of serous ovarian cancer associated with PID, but not with an earlier meta analysis of case control studies, which did not find an association between PID and either serous ovarian cancer or high grade serous ovarian cancer [42]. These discrepancies may be due to the fact that PID may be underreported in studies that rely on self report due to its sensitive nature, whilst only more severe or recurring episodes of PID may be captured in hospital records.

We found a 59% increased risk of HGSC in women who delivered twins and higher order multiples compared with women who did not, with confidence intervals that included one. Other authors have generally not found an association between ovarian cancer overall and the delivery of twins [33,37,39], although Albrektsen [32] found an increased risk of serous tumours in women who delivered twin girls. These different findings may be related to the fact that individual studies may not have sufficient power to adequately explore this association and because the association may differ according to ovarian cancer

subtype.

Our study has several strengths and limitations. A major strength was the implementation of a detailed pathology review of all ovarian tumours recorded in our cohort using up to date methodology and currently recognized classification schemes [46]. This review led to the re classification of a number of cases, particularly those previously classified as “not otherwise specified” and those of uncertain grade and mixed type. These unspecified (other) categories can make up as much as 30% of all ovarian cancer cases [13,31,52], and are predominantly high grade serous [46]. Other authors have emphasized the importance of histopathological review [2] and noted that without histopatholo gical review, estimates of risk may be less precise [59]. Strengths in clude the size of the study population; accurate recording of diagnoses and procedures in administrative databases rather than relying on personal report which could be subject to error and recall bias. Nevertheless, medical record linkage studies are not without flaws. Conditions under study had to have been diagnosed in hospital. Women who were not admitted to hospital for infertility, PID or endometriosis were classified as unexposed, even though some of these women would be suffering from these conditions.

The major limitation of this study was our inability to include in formation on oral contraceptive use. Use of oral contraceptives is as sociated with a significant reduction in ovarian cancer risk [60]. If women taking oral contraceptives were more, or less likely to be ex posed to the factors under study, then confounding may have resulted, and our risk estimates would be inaccurate. For example, if women diagnosed with PID were more likely to have taken oral contraceptives, then we may have underestimated the association between PID and HGSC. Another limitation of our study was the need to classify parity into two categories, with women who delivered zero, one or two chil dren compared with women who delivered three or more. We classified parity in this way to reduce misclassification of women who migrated into the state and only gave birth outside WA and women who only delivered children prior to 1970. A further limitation was the age of the study population. Because we wanted to capture information on ex posures that occurred early in a woman’s life, including births and tubal ligation, we needed to choose a relatively young study population. The average age at the end of follow up was 53 years, with the oldest women in the cohort aged 69 years.

4.1. Conclusions

Our finding that PID was associated with an increased risk of HGSC lends support to the hypothesis that inflammatory processes may play a role in the development of HGSC [40,61].

Funding

This work was supported by a grant from the Ovarian Cancer Research Foundation (OCRF).

Susan Jordan is supported by a fellowship from the National Health and Medical Research Council of Australia.

The funding source had no role to play in study design; in the col lection, analysis and interpretation of data; in the writing of the report; or in the decision to submit the article for publication.

Declaration of interest

LMS reports grants from Ovarian Cancer Research Foundation, during the conduct of the study.

KS, SJ, CS, CDJH, AP, JR and PC report no declaration of interest.

Authorship contribution

LMS and CS contributed to the conception and design and acquisi tion of data.

LMS and KS contributed to the analysis of data.

All authors contributed to the interpretation of data.

LMS drafted the article and all authors revised it critically for im portant intellectual content.

All authors approved the final version to be published.

Acknowledgements

The authors wish to thank the staff at the Western Australian Data Linkage Branch and staff and custodians of the Hospital Morbidity Data Collection, the WA Cancer Registry, the Midwives Notification System, Birth Registrations, Deaths Registrations and the WA Electoral Roll.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.canep.2018.05.011.

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